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INFLUENCIA DE LA MATRIZ VÍNICA Y DE PARÁMETROS ORO-FISIOLÓGICOS EN LA LIBERACIÓN DEL AROMA DEL VINO. IMPACTO EN EL AROMA RETRONASAL

CAROLINA MUÑOZ GONZÁLEZ

Tesis doctoral

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CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

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CAROLINA MUÑOZ GONZÁLEZ

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Dras. M. Ángeles Pozo-Bayón y M. Victoria Moreno-Arribas

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M. ÁNGELES DEL POZO-BAYÓN, CIENTÍFICA TITULAR DEL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS, Y Mª VICTORIA MORENO-ARRIBAS, INVESTIGADORA CIENTÍFICA DEL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS,

CERTIFICAN:

Que la memoria titulada "**Influencia de la matriz vínica y de parámetros oro-fisiológicos en la liberación del aroma del vino. Impacto en el aroma retronasal**", que presenta Dª. Carolina Muñoz González, para optar al grado de Doctor, se ha realizado bajo su dirección en el Departamento de Biotecnología y Microbiología de Alimentos del Instituto de Investigación en Ciencias de la Alimentación (CIAL), y como directoras de la misma autorizan su presentación.

Madrid, 6 de junio de 2014

Fdo.: Mª Ángeles del Pozo-Bayón

Fdo.: Mª Victoria Moreno-Arribas

C/ Nicolás Cabrera, 9.
Campus de la
Universidad Autónoma
de Madrid
28049 Madrid

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RESUMEN

El aroma es uno de los principales atributos de calidad de los vinos y está entre los más decisivos a la hora de explicar las preferencias de los consumidores. Por ello, en los últimos años, la mayor parte de los estudios científicos sobre este tema se han dirigido a la identificación y caracterización de los compuestos integrantes de la fracción volátil del vino. Estos compuestos debido a su baja presión de vapor se liberan fácilmente de la matriz vínica y son transportados durante la respiración a través de las fosas nasales, donde interaccionan con los receptores olfativos (vía ortonasal). Sin embargo, durante el consumo, los compuestos volátiles se liberan en el interior de la cavidad oral y llegan, gracias a los flujos respiratorios contracorriente a los receptores olfativos (vía retronasal). Esta diferencia de ruta de transporte de los compuestos volátiles hace que la percepción del aroma al oler o al consumir el vino, pueda ser muy diferente. A pesar del hecho de que el aroma retronasal está directamente relacionado con la percepción del aroma y es un factor decisivo en las preferencias de los consumidores, apenas hay estudios científicos encaminados a comprender el proceso de liberación del aroma durante el consumo de vino y su relación con la composición del aroma retronasal.

Por lo tanto, el objetivo de este trabajo ha sido determinar la influencia de distintos parámetros oro-fisiológicos (flujos respiratorios, saliva, microbiota oral, adsorción a mucosa) en la liberación de aroma retronasal durante el consumo considerando, a su vez, diferentes tipos de vino, y por tanto, diferencias en la composición de la matriz no volátil.

Para la consecución de este objetivo se ha empleado una amplia variedad de aproximaciones experimentales, tales como la monitorización del espacio de cabeza en estático que permitió comprobar el efecto de la matriz vínica en la liberación de aroma y que más tarde se confirmó en un estudio *in vivo* empleando para ello un sistema de atrapamiento del aroma retronasal que permitió obtener una representación del aroma exhalado por diferentes panelistas durante el consumo de vino. Como resultados más relevantes, se comprobó que la capacidad respiratoria de los individuos puede determinar en gran medida, la cantidad de aroma liberado durante el consumo. Además, mediante la técnica PTR-ToF-MS acoplada a una boca artificial se ha evaluado por

primera vez el efecto de la matriz vínica en la liberación de aroma en tiempo real, empleando condiciones que simulan la dinámica del proceso de consumo (incorporación de flujos de aire, saliva, temperatura controlada).

Entre los principales parámetros oro-fisiológicos que pueden influir en la composición del aroma retronasal, se comprobó mediante una aproximación *in vitro* empleando el análisis de espacio de cabeza en condiciones estáticas y dinámicas, que la saliva es un factor determinante que condiciona la liberación del aroma y que su efecto es diferente dependiendo de la composición no volátil de los vinos. La interacción de los compuestos del aroma del vino con la mucosa oral es otro de los factores relacionados con la fisiología del individuo, poniéndose de manifiesto en este trabajo que afecta a la liberación del aroma durante el consumo. En este sentido, se ha comprobado que la mucosa además de retener los compuestos del aroma, puede liberarlos con distintas cinéticas dependiendo de las características fisicoquímicas de los compuestos del aroma y la composición de la matriz. Finalmente, se evaluó el importante papel que la microbiota oral puede tener en la generación de compuestos odorantes a partir de precursores glicosídicos aislados de uvas.

En su conjunto, el presente trabajo ha proporcionado una información nueva y muy relevante sobre los parámetros orales que influyen en la liberación del aroma retronasal del vino, y en consecuencia, la información generada ayudará a comprender la percepción de aroma durante el consumo de vino y su relación con las preferencias de los consumidores.

SUMMARY

Aroma is one of the main attributes of wine quality and it is an outstanding aspect related to food preferences and choices. Therefore, in recent years, most of the studies on this topic have led to the identification and characterization of the aroma compounds presents in wines. These compounds due to their low vapor pressure are easily released from the wine matrix and passed through the nostrils where they interact with the odorant receptors (orthonasal pathway). However, during drinking, the volatile compounds are released into the oral cavity and, thanks to the airflows, they reach the olfactory receptors (retronasal pathway). This difference in the transport route provokes that the perception when we smell or consume a wine could be very different. In spite of the facts that retronasal aroma is directly related with flavour perception, and it is a key modulator for food consumption and food preferences, the works focused in understanding the aroma release during wine consumption are almost inexistent.

Therefore the main objective of this work, has been to determine the influence of different oro-physiological factors (breathing flows, saliva, oral microbiota, adsorption to oral mucosa) on the retronasal aroma delivery during wine consumption taking into consideration different wine types (different nonvolatile wine matrix composition).

For this work a wide array of experimental approaches has been used. First of all, the impact of wine matrix composition on aroma release has been tested by using static conditions (HS-SPME-GC/MS). This effect was confirmed latter by following an *in vivo* approach using a retronasal trapping system to obtain breath extracts during wine drinking. This work showed the importance of the breathing capacity on aroma release from panelists. Besides that, PTR-ToF-MS coupled to an artificial mouth allowed evaluate the effect of wine matrix composition on aroma release in real time, by applying dynamic conditions that simulate the dynamics of the consumption process (incorporation of airflows, saliva, temperature controlled).

Among the main orophysiological parameters that can influence retronasal aroma composition, the impact of saliva on wine aroma release by using *in vitro* approaches (static and dynamic headspace conditions) have been tested. It was found that saliva is a factor that determines the flavor release and its effect is different depending on the wine matrix composition. In addition, the impact of oral mucosa on

aroma retention by using *in vivo* conditions was proved. In this regard, it has been found that mucosa retained aroma compounds that can be released with different kinetics depending on the physicochemical characteristics of the aroma compounds and matrix composition. Finally, the important role that oral microbiota can have on the generation of odorant compounds from glycosidic precursors isolated from grapes was also evaluated.

Overall, this work has provided new and relevant information about the oral parameters that influence retronasal aroma from wine, which might help in understanding wine aroma perception during wine consumption and its relationship with consumer's preferences.

Antecedentes bibliográficos

1. ANTECEDENTES BIBLIOGRÁFICOS

1.1 El aroma del vino

El aroma es uno de los principales atributos de calidad de los alimentos y está entre los más decisivos a la hora de explicar las preferencias de los consumidores. En el caso del vino es una característica esencial ya que se trata de un alimento de alto valor añadido del que el consumidor espera obtener un elevado placer sensorial.

El aroma del vino es el resultado de una larga secuencia de transformaciones químicas y bioquímicas, que comienzan en el propio grano de uva con la síntesis de precursores de aroma y de moléculas de carácter varietal (aroma primario) que van a tener un gran impacto en el aroma final. Durante las fermentaciones alcohólica y maloláctica siguen generándose nuevos compuestos aromáticos dependientes del tipo de microorganismo presente y de las condiciones de fermentación (aroma secundario). Por último, el aroma terciario también denominado "*bouquet*", está formado por los compuestos volátiles que se han formado durante la etapa envejecimiento en barrica y de maduración en botella de los vinos. Las diferentes variedades de uva, condiciones de cultivo, factores ambientales, microorganismos implicados y la tecnología de elaboración empleada son factores que van a tener una incidencia directa en el aroma final y dependiendo de la combinación de estas variables se puede obtener un amplio espectro de vinos con características aromáticas muy diferentes.

La importancia del aroma en la calidad final de un vino ha suscitado un gran interés científico y como resultado de ello, numerosos trabajos científicos se han encaminado a conocer e identificar la compleja fracción volátil del vino, por lo que ya a finales de los años ochenta se habían identificado más de 800 compuestos volátiles (Maarse y Vischer, 1989). Desde el punto de vista químico, el aroma está constituido por compuestos orgánicos que poseen bajos pesos moleculares (< 300 Da) y altas presiones de vapor (compuestos de alta volatilidad), lo que facilita su liberación de la matriz en la que se encuentran. Otras características de este tipo de compuestos son: su gran heterogeneidad en cuanto a estructura química (ésteres, aldehídos, cetonas, lactonas, terpenos, ácidos, compuestos azufrados), características físico-químicas (volatilidad, polaridad), y el amplio rango de concentraciones en las que aparecen en los vinos (desde mg/L a ng/L). Muchos de los trabajos encaminados a la caracterización de este tipo de compuestos se han recogido en distintas revisiones bibliográficas (Rapp y

Mandery, 1986; Polásková y col., 2008; Ebeler y Thorngate, 2009; Styger y col., 2011, Muñoz-González y col., 2011, Villamor y Ross, 2013; etc.).

Al tratarse de moléculas volátiles, la cromatografía de gases (GC) se ha convertido en la técnica de elección para la separación y posterior análisis de estos compuestos. En este sentido, el uso de la clásica cromatografía unidimensional se ha combinado en los últimos años con el empleo de la cromografía bidimensional (GC x GC) que proporciona un mayor poder de resolución, mejorando la separación en muestras complejas, como es el caso del vino (Muñoz-González y col., 2011). La rapidez en la elución y la elevada resolución de los picos cromatográficos derivada del empleo de estas técnicas también ha requerido el desarrollo de detectores diferentes a los tradicionales (detector de ionización de llama (FID)), como los espectrómetros de masas (MS) de doble o triple cuadrupolo o acoplados a analizadores de tiempo de vuelo (ToF-MS) que permiten escanear de manera más rápida y sensible los analitos eluidos. Pero sin duda alguna, la evolución en las técnicas de preparación de muestra ha sido el gran hito que ha permitido profundizar en el conocimiento de la fracción volátil del vino. El empleo de técnicas como la extracción líquido-líquido (LLE, Liquid-Liquid Extraction) (Ferreira y col., 1993; Ferreira y col., 1998; Ortega-Heras y col., 2002; Andújar-Ortiz y col., 2009), la extracción en fase sólida (SPE, Solid Phase Extraction) (López y col., 2002; Hernanz y col., 2008), la microextracción en fase sólida (SPME, Solid Phase Microextraction) (Mestres y col., 1998; Ezquerro y col., 2003; Ezquerro y col., 2004; Perestrelo y col., 2009; Pozo- Bayón y col., 2001), la extracción por sorción sobre barra agitadora (SBSE, Stir Bar Sorptive Extraction) (Coelho y col., 2009), entre otras, han permitido el aislamiento y concentración de los compuestos volátiles que se encuentran en el vino en pequeñas cantidades (incluso a niveles traza), permitiendo la identificación tanto de compuestos que contribuyen al aroma positivo del vino, como a defectos aromáticos (*off flavor*). Además, en los últimos años, muchas de estas técnicas han evolucionado y mejorado para intentar alcanzar una mayor exactitud, precisión, sensibilidad, rapidez y una reducción en el coste y en la cantidad de solventes orgánicos empleados (Muñoz-González y col., 2011).

Sin embargo, como apuntó el científico francés Étievant a principios de los años noventa, el conocimiento analítico dirigido a descifrar el aroma del vino todavía no había sido capaz de interpretar el papel que ejercía cada componente individual en el

mismo (Ferreira 2002). Y es que a pesar de que en la actualidad se conoce la existencia de más de mil compuestos volátiles presentes en el vino (Polásková y col., 2008), sólo un número limitado de los mismos puede ser considerado olfativamente activo, lo que depende de su concentración y de su umbral de percepción (concentración mínima de un compuesto necesaria para ser detectados por el olfato humano).

En este sentido, las investigaciones desarrolladas en los últimos veinte años se han dirigido a estudiar el significado sensorial de las moléculas aromáticamente activas, es decir, aquellas que desempeñan un papel relevante en la percepción final del aroma de un vino (**Figura 1**). Para ello, se han aplicado técnicas analíticas en combinación con el olfato humano como la cromatografía de gases acoplada a la detección olfatométrica (GC-O) y experimentos de omisión-reconstitución que han permitido comprobar que tan sólo unos pocos compuestos (entre 40 ó 50) tienen un verdadero impacto en el aroma del vino (Ferreira 2002).

En el análisis GC-O los compuestos separados que eluyen de la columna cromatográfica son sensorialmente evaluados por una persona (sniffer), empleando la nariz como un detector mucho más sensible que cualquier detector analítico conocido. A partir del análisis GC-O se puede obtener el denominado valor de actividad de aroma (OAV), que es el ratio entre la concentración de un compuesto en el vino y su umbral de detección. Generalmente se considera que compuestos con OAV > 1 tienen impacto sensorial, tanto mayor cuanto mayor sea este valor.

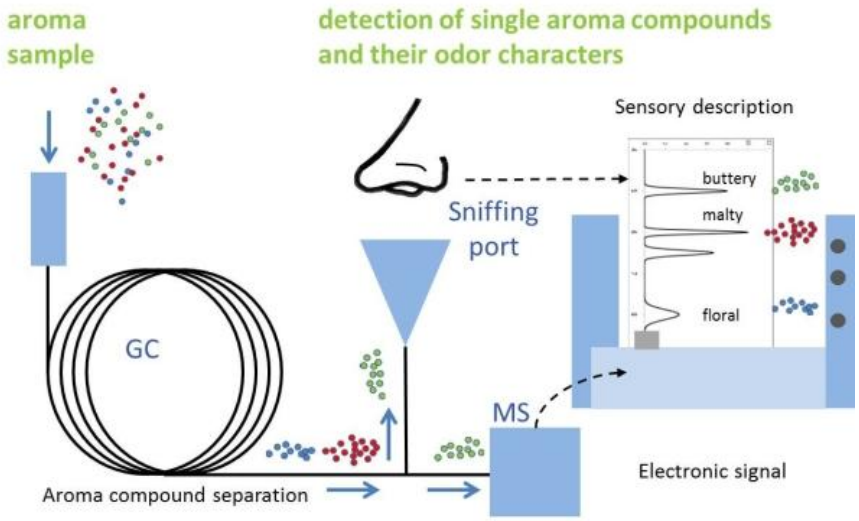


Figura 1. Esquema del análisis mediante GC-O.
(http://www.foodprocessing-technology.com/contractors/training/flavologic/flavologic2.html)

Para trabajar con esta técnica, se han propuesto diferentes metodologías, como la técnica AEDA (Aroma Extract Dilution Analysis) (Ullrich y Grosch, 1987), el método Charm ® (Acree y col., 1984), la técnica OSME (Odor Specific Magnitude Estimation) (McDaniel y col., 1990) o más recientemente el método de Frecuencia de Detección (Pollien y col., 1997).

Sin embargo, a pesar de la indudable utilidad de este tipo de metodología para jerarquizar la importancia odorante de los compuestos volátiles que integran el aroma de un vino, la técnica GC-O presenta algunas limitaciones. La más importante se debe a que en GC-O los compuestos son primero separados y la calidad odorante de cada uno de ellos es evaluada independientemente y no de forma integrada como ocurre cuando se percibe el aroma de un vino. De este modo, no se tienen en cuenta los fenómenos de interacción con los componentes no volátiles de la matriz vínica y de aditividad, sinergismo o antagonismo con otros compuestos volátiles presentes en la mezcla (Ferreira 2002; Pozo-Bayón y Reineccius, 2009; Lytra y col., 2013). La segunda limitación se refiere a la representatividad del extracto obtenido y que es posteriormente evaluado por GC-O. Recientemente d'Acampora y col. (2008) y Ferreira y col. (2009) han descrito las ventajas e inconvenientes del empleo de las diferentes técnicas preparativas para obtener un extracto representativo que refleje la composición aromática de partida.

El empleo de los ensayos de omisión-reconstitución puede solventar estos problemas. En este tipo de análisis, se realiza un primer screening mediante GC-O en el que se detectan los compuestos odoríficamente activos y, posteriormente, se prepara una solución sintética con todos ellos (experimento de reconstitución) (Grosch 1993). A continuación, en los ensayos de omisión los distintos componentes de la mezcla se van eliminando uno a uno con el objetivo de medir y verificar cuál es el efecto sensorial que la eliminación del componente tiene sobre el aroma global. Pese a que este método ha funcionado muy bien en el caso de vinos con un fuerte aroma varietal (Guth, 1997; Ferreira y col., 2002; Escudero y col., 2004), es más problemático en ensayos dirigidos a interpretar el análisis de vinos más complejos (Ferreira 2002).

De forma paralela al desarrollo de las técnicas analíticas, las técnicas de análisis sensorial han evolucionado para proporcionar datos sensibles, precisos y exactos sobre

las características sensoriales de los vinos (Ebeler 2005). Sin embargo, los avances en ambas técnicas están lejos de poder relacionar la composición global de un vino con sus propiedades sensoriales (Styger y col., 2011). Esto puede ser debido a la existencia de compuestos aromáticos aún por identificar en el vino debido a las limitaciones de las actuales técnicas analíticas, o también a otros fenómenos como la interacción de las moléculas del aroma con los componentes no volátiles del vino (matriz vínica), o a la transformación de los mismos durante el proceso de consumo debido a diferente mecanismos físico-químicos y fisiológicos que tienen lugar durante este proceso. Además, los compuestos del aroma pueden interaccionar a nivel cognitivo, provocando fenómenos de aditividad, sinergismo o antagonismo (Atanasova y col., 2005; Roudnitzky y col., 2011), modificando la percepción perceptual.

1.2 Liberación y percepción del aroma durante el consumo

La percepción de aroma es un proceso multimodal y dinámico en el que intervienen tanto los sentidos del olfato y gusto, como el sistema somatosensorial (Reineccius 2006). Además la percepción del aroma depende de otros factores como factores cognitivos y culturales, entre otros.

El primer contacto que el consumidor tiene con el aroma de un producto es el que se produce como consecuencia del desprendimiento de las moléculas odorantes contenidas en la matriz alimentaria que entran directamente a través de las fosas nasales al epitelio olfativo (vía ortonasal). Cuando se produce la unión entre los receptores olfativos localizados en este epitelio y una molécula odorante se origina una cascada de señales mediadas por proteínas G en la que la señal viaja por el nervio olfativo hasta el cerebro donde se produce el reconocimiento del estímulo sensorial conocido como ‘olor’ (**Figura 2**).

Durante la ingestión de un alimento, es decir en condiciones de consumo, el aroma se libera de la matriz alimentaria dentro de la cavidad oral. En una primera etapa, el aroma contenido en los alimentos pasa a la fase líquida (saliva) que lo rodea, y desde aquí a la fase gaseosa, a partir de la cual será transportado vía retronasal a los receptores olfativos siguiendo el mismo proceso de interacción con los receptores olfativos, anteriormente mencionado (Hills y Harrison, 1995). Este tipo de aroma se conoce como aroma retronasal y sólo se experimenta durante el consumo de alimentos (**Figura 2**).

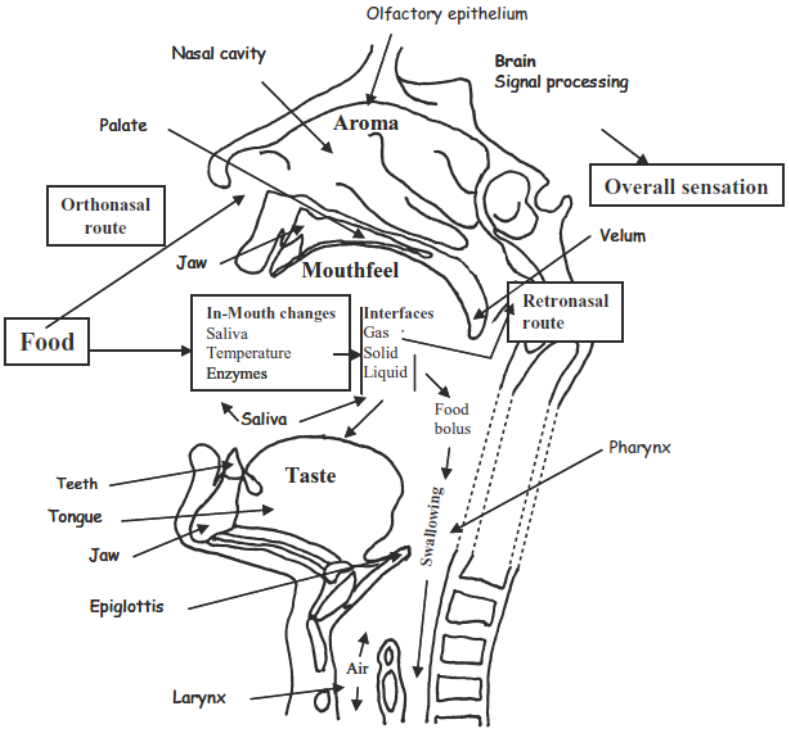


Figura 2. Mecanismo de liberación y percepción del estímulo sensorial durante el consumo de alimentos. (Fuente: Gierczynski y col., 2011).

Para que se produzca la percepción retronasal, la cavidad oral y nasal han de estar comunicadas. En situación de reposo, la boca es un sistema estanco constituido por una barrera anterior formada por los labios y una barrera posterior formada por el velo del paladar y la base de la lengua. Este cierre impide que los alimentos fluyan hacia la faringe hasta que se produzca el acto de la deglución, y su apertura provoca que los compuestos volátiles puedan viajar de la cavidad oral a la nasal (donde se encuentran los receptores olfativos), siendo este el mecanismo más importante por el que los componentes del aroma de un alimento son percibidos. La deglución consiste en forzar que el bolo alimentario entre en la faringe, mientras que el velo del paladar se retrae y eleva, impidiendo que el alimento entre en la cavidad nasal. El evento de la deglución se produce en cuatro fases (Guinard y Mazzucchelli, 1996; Hodgson y col., 2003). La fase preparatoria (procesamiento del alimento en la boca, masticación), la fase voluntaria (la lengua propulsa el bolo hacia la parte posterior de la cavidad oral iniciándose la deglución reflexiva), la fase faríngea (el bolo alimentario se transfiere a la faringe), y

por último, la cuarta fase en la que por movimientos peristálticos esofágicos el bolo alimentario alcanza el estómago, para continuar el proceso digestivo. Durante la fase faríngea de deglución, la laringe sube y comprime la epiglotis, que es la encargada de cerrar la entrada a la tráquea, y de este modo se evita que el alimento penetre en las vías respiratorias (Buettner y col., 2001). Tras la deglución, la mayor parte del alimento desaparece por el esófago pero en su paso por la faringe se forma una fina capa viscosa que la recubre a lo largo de su superficie. Este hecho, fue recientemente visualizado por Buettner y col., (2002a) mediante el uso de herramientas médico-analíticas como la videofluoroscopía e imagen por resonancia magnética a tiempo real (**Figura 3**).

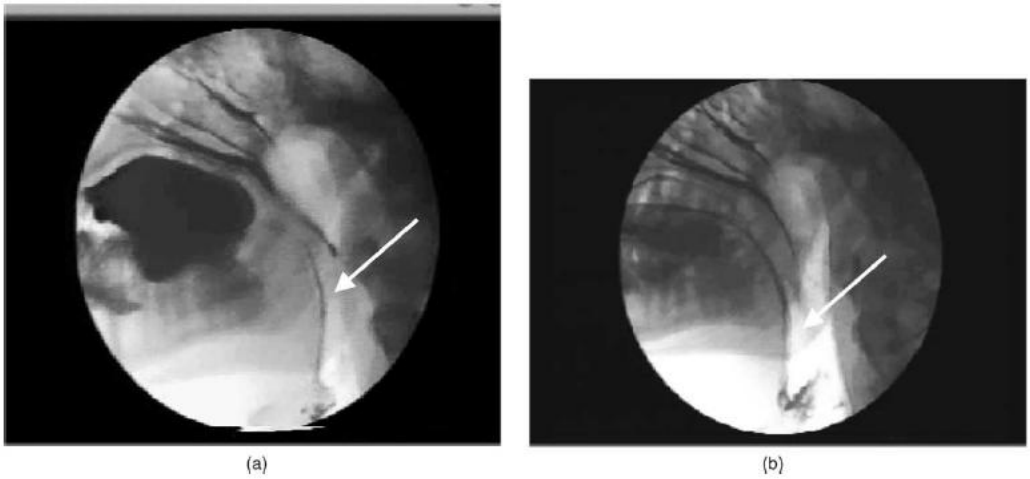


Figura 3. Observación de la formación de un recubrimiento viscoso por videofluoroscopía después del consumo de un alimento semisólido: (a) con el alimento presente en la cavidad oral, y (b) durante la deglución del alimento (Fuente: Buettner y col., 2002a)

Durante la exhalación inmediatamente posterior a la deglución, se crea un gradiente de concentración de aroma entre la capa que recubre la superficie de la faringe y el aire exhalado que pasa a su través. Ese aire exhalado entra dentro de la cavidad nasal y alcanza el epitelio olfativo donde los compuestos de aroma son percibidos vía retronasal, en un pulso de aroma conocido como “swalow breath” (Land 1996; Buettner y Schieberle, 2000; Linforth y Taylor, 2000; Linforth y col., 2002; Linforth y Taylor, 2006).

1.2.1 Aproximaciones analíticas para el estudio de la liberación del aroma durante el consumo de vino

El estudio de la liberación de aroma durante el consumo se puede abordar mediante el empleo de diferentes estrategias: técnicas de análisis sensorial, analíticas u otras como el empleo de sensores o técnicas de modelización. A continuación se repasan brevemente cada una de ellas.

1.2.1.1 Análisis sensorial

El Instituto de Tecnólogos de Alimentos (IFT, Chicago, EEUU) define la evaluación sensorial como la disciplina científica que "evoca, mide, analiza e interpreta las reacciones a las características de los alimentos, que se han percibido por los sentidos de la vista, olfato, gusto, tacto y oído". Las técnicas tradicionales de análisis sensorial estáticas han dado paso en los últimos años a técnicas dinámicas que permiten medir la evolución de las sensaciones a lo largo del tiempo. Por tanto, estas técnicas son idóneas para medir la liberación de aroma durante el consumo. De entre ellas, las técnicas basadas en los perfiles de tiempo-intensidad (TI) son las más ampliamente empleadas, ya que proporcionan datos analíticos objetivos (máxima intensidad de la sensación, tiempo de duración, etc.) a partir de sensaciones subjetivas, por lo que permiten comparar de manera objetiva distintos productos, diferencias interindividuales, etc. Esto ha permitido incluso establecer modelos teóricos predictivos de la percepción sensorial temporal que se experimenta en el transcurso del consumo de alimentos (Lee y Pangborn, 1986; Overbosch 1986).

No obstante, la existencia de interacciones entre las diferentes modalidades sensoriales (por ejemplo entre el sabor y el aroma ver **Figura 4**) y otros factores, como son el elevado tiempo requerido debido a las sesiones de entrenamiento previo, el difícil tratamiento de datos (Gierczynski y col., 2011), u otros relativos al agrado o disgusto a la hora de evaluar el aroma de un alimento, pueden afectar notablemente su evaluación (Ross 2009).

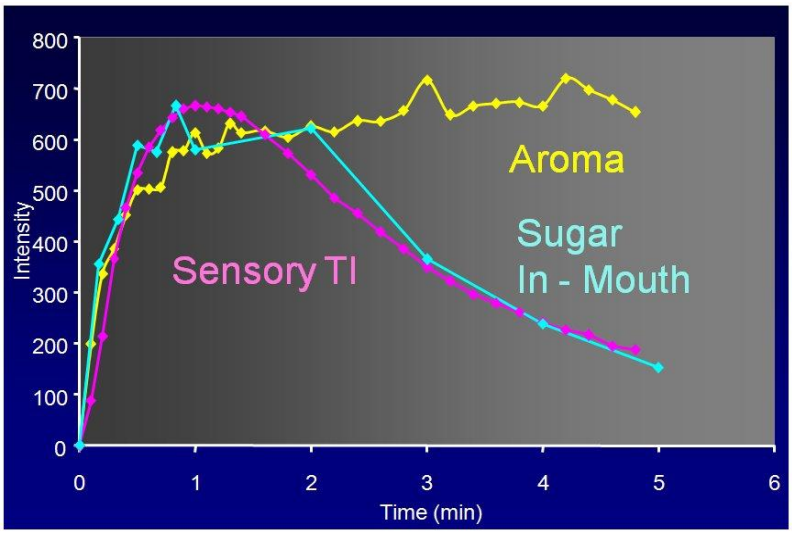


Figura 4. Ejemplo de perfil Tiempo-Intensidad (http://www.flavometrix.co.uk/MSnose.html)

Es de destacar que Pineau y colaboradores (2009) desarrollaron una técnica dinámica tiempo-intensidad llamada "dominio temporal de las sensaciones" (TDS, Temporal Dominance Sensations) que permite el estudio de la evolución de varios atributos y sus interacciones durante el consumo, mejorando la discriminación entre productos en comparación con la tradicional TI. Este método se ha utilizado para describir el impacto de la desalcoholización parcial por ósmosis inversa en la percepción sensorial de vinos Merlot y Syrah y su relación con las preferencias de los consumidores (Meillon y col., 2009; Meillon y col., 2010).

A pesar de la relevancia de las técnicas sensoriales para determinar las sustancias odorantes percibidas durante el consumo de un alimento, para llegar a caracterizar los compuestos responsables de una determinada sensación se requiere del empleo de técnicas analíticas que estudien el aroma liberado durante el consumo.

1.2.1.2 Técnicas analíticas

El desarrollo de instrumentación para obtener datos que reflejen mejor los perfiles de liberación de volátiles desde el alimento a los receptores olfativos ha supuesto un enorme avance a la hora de entender el proceso de liberación de aroma durante el consumo. La mayoría de las técnicas se pueden agrupar en modalidades *in vitro* o *in vivo*.

1.2.1.2.1 Análisis del aroma retronasal por métodos in vitro

En general, las aproximaciones analíticas empleadas para simular la liberación de aroma durante el consumo de alimentos se han centrado en el empleo de diferentes dispositivos *in vitro*. Estas aproximaciones analíticas, se basan principalmente en el empleo de técnicas de espacio de cabeza tanto en condiciones estáticas como dinámicas que permiten cuantificar los compuestos que se liberan al espacio de cabeza del alimento. En muchos casos, se ha tratado de mimetizar en la medida de lo posible los procesos fisiológicos que acontecen durante el consumo de alimentos para simular el proceso de liberación del aroma que ocurre en una situación *in vivo*.

Para ello, se han empleado distintos dispositivos, más o menos sofisticados, (bocas artificiales, gargantas artificiales, etc.) que permiten monitorizar la liberación de los compuestos del aroma que se liberan del alimento y que pueden ser posteriormente recogidos en diferentes trampas (polímeros adsorbentes, trampas criogénicas, etc.) para su posterior análisis por cromatografía de gases o directamente por espectrometría de masas (Piggott y Schaschke, 2001). Estos sistemas son muy útiles, ya que permiten entender mejor las contribuciones de cada factor que afecta al proceso de consumo de manera individual, lo que sería muy difícil de evaluar en un estudio sensorial directo. Además, proporcionan un estricto control de las variables de estudio y permiten llevar a cabo un gran número de experimentos evitando las posibles diferencias inter-individuales. Sin embargo, muchos de estos dispositivos no tienen en cuenta algunos procesos importantes que tienen lugar durante la situación real del consumo (como el efecto de las mucosas bucal o faríngea, la posible actividad enzimática de la saliva o de los microorganismos presentes en la cavidad oral etc.), ni permiten captar la temporalidad del proceso de liberación del aroma. Sobre este tema, existen varias revisiones bibliográficas al respecto (Stephan y col., 2000; Piggott y Schaschke, 2001; Salles y col., 2011; Morell y col., 2014).

La aproximación más sencilla para monitorizar el aroma liberado de un alimento durante el consumo, consiste en utilizar una técnica tradicional de espacio de cabeza en estático en la que se pueden prefijar unas condiciones de temperatura, normalmente cercanas a la temperatura corporal (37 ºC) y evaluar el efecto de un determinado parámetro oro-fisiológico. En este sentido, Mitropoulou y colaboradores (2011) utilizaron SPME-GC/FID para evaluar el efecto de la saliva artificial en la liberación de

aroma en vinos modelo dopados con diferentes concentraciones de polisacáridos y polifenoles. En este trabajo observaron que la presencia de estos macrocomponentes producía, en general, un aumento de la volatilidad de los compuestos más hidrofóbicos mientras que los hidrofílicos fueron más retenidos en la matriz.

En los últimos años se han desarrollado numerosos prototipos más complejos que el anteriormente descrito. Estos dispositivos, reciben el nombre de bocas artificiales y han ido perfeccionándose e incorporando nuevas variables, como la presencia de dientes e incluso con control informático para simular al máximo el proceso de masticación, que es un aspecto crítico en el caso de alimentos sólidos (van Ruth y col., 1994, van Ruth y Roozen, 2000; Deibler y col., 2001; Salles y col., 2007; Arvisenet y col., 2008; Poinot y col., 2009; Charles y col., 2013). Sin embargo, el empleo de estos dispositivos ha proporcionado en general mejores resultados cuando se ha aplicado el estudio de la liberación de aroma en alimentos líquidos (Poinot y col., 2009), ya que en este caso no es necesario considerar el complejo proceso mecánico de la masticación. Algunos de estos ejemplos han sido las bocas artificiales diseñadas por Margomenou y col. (2000), Rabe y col. (2002), Rabe y col. (2004a), etc.

En el caso del vino, la bibliografía relacionada con el uso de bocas artificiales es prácticamente inexistente. Sin embargo, recientemente Genovese y colaboradores (2009) investigaron el efecto de la saliva (humana y artificial) en la liberación de volátiles de un vino blanco y otro tinto empleando un dispositivo que simula una boca artificial conectado *off line* con HS-SPME-GC y HS-SPME-GC/MS empleando condiciones dinámicas. El trabajo de Genovese ha sido el primero en utilizar este tipo de dispositivos para simluar el aroma retronasal del vino y sus resultados mostraron un efecto significativo de la saliva. Además sugirieron un importante efecto de las enzimas salivares (lipasa, esteresa, peroxidasa) y de la mucina sobre el aroma del vino. Por otra parte, observaron que el tipo de compuesto de aroma (clase química) y composición de la matriz (contenido en polifenoles) podría afectar el grado de este efecto. Sin embargo, en este estudio, no se tuvo en cuenta el diferente contenido de etanol de los tipos de vinos ensayados, que tiene un gran impacto en el coeficiente de partición de los compuestos volátiles, ni tampoco consideraron las diferencias en la concentración inicial de aromas en ambos tipos de vinos. Además, las muestras de saliva que utilizaron contenían microorganismos, por lo tanto, es difícil elucidar si los cambios se deben a la presencia de microorganismos o a la actividad enzimática de la saliva. Por

otro lado, tampoco se extrajeron conclusiones sobre los compuestos no volátiles de la matriz del vino responsables del efecto observado. Estos factores podrían haber afectado la discusión de los resultados. No obstante, y a pesar de todas estas limitaciones hay que remarcar que este trabajo es uno de los pioneros en reconocer el efecto de factores relacionados con la fisiología oral en la composición del aroma retronasal del vino.

Por otro lado, en los últimos años se ha comprobado que, sobre todo en alimentos líquidos, la deglución juega un importante papel en la percepción de aroma (De Roos y Wolswinkel, 1994). Como se comentó anteriormente (**Apartado 1.2**), tras la deglución de un alimento se forma una fina capa viscosa que recubre la faringe y se ha observado que la mayor parte de aroma depositado en esa capa se libera casi instantáneamente durante la primera exhalación tras el consumo (swallow breath) (Buettner y col., 2001; Weel y col., 2003). Por ello, también se han desarrollado sistemas basados en gargantas artificiales, priorizando los procesos que tienen lugar tras la deglución, y que pueden ser adecuados para mimetizar la liberación de aroma durante el consumo de alimentos líquidos (Weel y col., 2004; King y col., 2006; Pozo-Bayón y col., 2009a).

El empleo de técnicas espectrométricas como la APCI-MS (Atmospheric Pressure Chemical Ionization) (Weel y col., 2004; King y col., 2006; Pozo-Bayón y col., 2010) o la PTR-MS (Proton Transfer Mass Spectrometry) (Pozo-Bayón y col., 2009a; Buettner y col., 2008), acoplados a bocas o gargantas artificiales *in vitro* permiten monitorizar de manera continua la liberación del aroma en tiempo real (Yven y col., 2010), y por lo tanto, captar la dimensión temporal del aroma de los alimentos proporcionando una mejor correlación entre la sensación percibida durante el consumo y la concentración y el tipo de molécula responsable.

Todos los dispositivos descritos anteriormente, son una herramienta de gran utilidad a la hora de estudiar los efectos de parámetros orofisiológicos de manera independiente, pero no siempre permiten controlar los procesos tan complejos que se producen en condiciones reales de consumo y por lo tanto, muchas veces, resulta complicado establecer una relación directa entre estos datos y los proporcionados mediante análisis sensorial (Piggott y Schaschke, 2001; Taylor 2002; Rabe y col., 2004b). Para simular de manera conjunta todos los eventos que ocurren durante el

consumo de alimentos, sería deseable que el dispositivo ideal integrara numerosas funciones y en consecuencia, que fueran mucho más complejos que los descritos hasta la fecha.

1.2.1.2.2 Análisis in vivo

En la actualidad, la posibilidad de integrar todos los procesos fisiológicos que ocurren durante el consumo de un alimento solo es posible mediante el análisis *in vivo*. La liberación del aroma durante el consumo se puede medir muestreando los compuestos volátiles exhalados directamente en la boca o la nariz del consumidor con el objetivo de proporcionar una mejor representación de los compuestos volátiles que alcanzarán el epitelio olfativo (Piggott y Schaschke, 2001). Este tipo de metodología ha recibido diferentes nombres: análisis del aroma *in vivo*, "nosespace" o "breath by breath" (Taylor y Linforth, 2000; Yeretzian y col., 2000; Taylor y Linforth, 2003).

Un importante punto a tener en cuenta durante el empleo de esta metodología es la gran variación entre individuos, debido a diferencias en flujos respiratorios, patrones de deglución, composición de la saliva, etc (Buettner y col., 2001; Taylor 2002; Normand y col., 2004; Mestres y col., 2006). Este problema, sin embargo, puede reducirse empleando un amplio número de individuos, usando datos normalizados y siguiendo estrictos procedimientos de consumo (Piggott y Schaschke, 2001, Normand y col., 2004; Aprea y col., 2007; Salles y col., 2011). De hecho, en un reciente estudio llevado a cabo por Aprea y colaboradores (2007) el uso de un protocolo de consumo permitió reducir la variabilidad interindividual en un 52%.

La monitorización de liberación de aroma durante el consumo puede realizarse en tiempo real o de manera *off line*. Respecto a esta segunda aproximación, se han descrito varios sistemas de atrapamiento de aroma, con una característica común: los volátiles se atrapan durante el consumo gracias al uso de trampas poliméricas que posteriormente pueden desorberse y analizarse por cromatografía gaseosa (Linforth y Taylor, 1993; Delahunty y col., 1996). Estos dispositivos permiten un enriquecimiento de la muestra que normalmente está muy diluida, una separación previa ya que pueden acoplarse a GC-O y GC-MS y por tanto, una identificación más exacta. Además, pueden proporcionar una mayor selectividad por el uso de diferentes materiales poliméricos y son relativamente fáciles de instalar en cualquier laboratorio.

Existen varios tipos de sistemas de atrapamiento en función de si los volátiles exhalados se monitorizan a nivel de la cavidad nasal u oral. El primer grupo de sistemas de atrapamiento son los que muestrean los volátiles presentes en la cavidad oral y se basan en la premisa de que los volátiles monitorizados a este nivel se encuentran en una concentración similar a la que alcanzan en los receptores olfativos (O'Riordan y Delahunty, 2001). Roozen y Legger-Huysman (1994) desarrollaron un sistema mediante el cual los compuestos volátiles liberados en la boca tras el consumo eran atrapados en una trampa de Tenax empleando vacío y posteriormente fueron analizados por GC. Este sistema se conoce como "muestreo de la respiración oral" (OBS, Oral Breath Sampling), y fue posteriormente modificado y adaptado para estudiar la liberación del aroma de café en diferentes bebidas con distinto contenido en grasa y proteínas (Denker y col., 2006) (**Figura** 5).

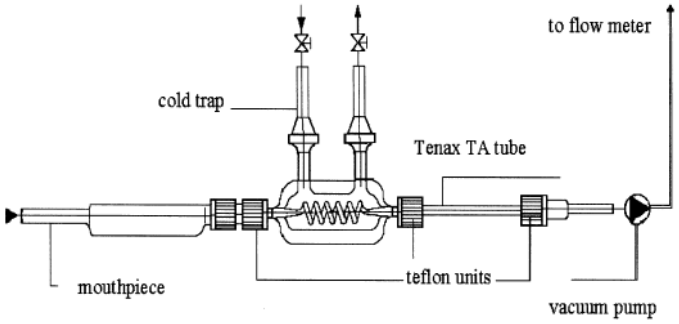


Figura 5. Muestredor de la respiración oral de Roozen y Legger-Huysman, 1994 (Fuente: Stephan y col., 2000).

La prolongada percepción del aroma retronasal, a menudo llamada persistencia del aroma puede ser debida a la liberación de los volátiles absorbidos por las mucosas oral o faríngea después del consumo de alimentos. La mucosa puede actuar como una especie de depósito aromático, responsable de la persistencia en el tiempo de algunos compuestos volátiles después del consumo (Buettner 2004). La adsorción de los odorantes a la mucosa oral puede evaluarse de diferentes maneras. Una de ellas, consiste en calcular la diferencia entre la cantidad de aroma presente en una solución que ha sido mantenida en la boca durante cierto tiempo y posteriormente expectorada (SOOM, Spit Off Odorant Measurement). Esta aproximación fue empleada por Buettner y col., (2002a) quienes unos años más tarde desarrollaron otro sistema que permitía

monitorizar la liberación de aroma en la cavidad bucal (BOSS, Buccal Odour Screening System). Esta técnica se basa en la extracción intraoral a tiempos definidos de compuestos odorantes que se han quedado retenidos en la cavidad bucal gracias al empleo de una barrita agitadora (SBSE o Twister). Seguidamente los volátiles son desorbidos en la unidad de desorción térmica y analizadas por GC-O o GC-MS (Buettner 2004; Buettner y Welle, 2004; Buettner y Mestres, 2005).

El segundo grupo de sistemas de atrapamiento de aroma es el basado en el muestreo en la cavidad nasal (análisis in-nose) (Ingham y col., 1995). Estas técnicas analíticas también se basan en la premisa de que la concentración de aroma que se alcanza en las fosas nasales es similar a la que llega a los receptores olfativos (Taylor 1996; Denker y col., 2006; Linforth y col., 2002), aunque recientemente se considera la posibilidad de la existencia de un gradiente intranasal que produzca variaciones temporales en la concentración dependiendo del tipo de compuesto (Frasnelli y col., 2005).

Los primeros análisis in-nose se basaron en el atrapamiento de los volátiles liberados a través de las fosas nasales en una trampa polimérica o criogénica a diferentes tiempos después del consumo de alimentos (Piggott y Schaschke, 2001). En este sentido, Buettner y Schieberle (2000) introdujeron el concepto de la "medida del odorante exhalado" (EXOM, Exhaled Odorant Measurement) para obtener datos cuantitativos y precisos de liberación de aroma de alimentos. Esta técnica combinaba las ventajas del atrapamiento en un material adsorbente como el Tenax junto con la aplicación del "ensayo de dilución de isótopos estables" (SIDA, Stable Isotope Dilution Assay), permitiendo una cuantificación muy exacta de los volátiles de la respiración. Además, ofrecía la posibilidad de concentrar los odorantes antes del análisis, por lo tanto, fue una aproximación útil para estudiar la liberación de aroma en las concentraciones tan bajas en las que se encuentran muchos de los odorantes en condiciones de consumo reales *in vivo*, que no podrían detectarse usando las técnicas de análisis en tiempo real. Además, con este procedimiento se evaluó la globalidad de los factores orofisiólogicos que influían en el proceso de consumo de alimentos, incluyendo el proceso de deglución. Esta metodología ha sido recientemente utilizada por Lasekan y col. (2009) para evaluar la liberación de aroma durante el consumo de una bebida fermentada conocida como "vino de palma". Sin embargo, hasta nuestro conocimiento

estos sistemas no se han aplicado para evaluar la liberación del aroma durante el consumo de vino.

No obstante, estas técnicas se basan en el atrapamiento del total de los compuestos volátiles liberados durante el consumo y no toman en consideración la dimensión dinámica de la liberación de aroma que se produce durante el consumo, y que podría correlacionar mejor con la evolución de la percepción del aroma de los alimentos a lo largo del consumo.

El análisis de aroma en tiempo real es una aproximación metodológica muy importante para establecer la relación entre liberación y percepción de aroma durante el tiempo que dura el consumo de un alimento (Linforth y Taylor, 1993; Avison 2013). El análisis en tiempo real es posible gracias al empleo de técnicas espectrométricas, tales como APCI-MS (Taylor y Linforth, 1996), PTR-MS (Lindinger y col., 1998; Yereztian y col., 2000) y SIFT-MS (Spanel y Smith, 1999). Entre ellas, las más ampliamente utilizadas son APCI-MS y PTR-MS. La primera fue desarrollada para el análisis de aromas y fragancias mientras que el análisis de contaminantes atmosféricos o la monitorización de volátiles de respiración con fines médicos fue el objetivo principal de la segunda. Sin embargo, en la actualidad ambas técnicas se utilizan indistintamente en estudios de liberación del aroma durante el consumo.

Estas técnicas se basan en la monitorización de los compuestos del aroma en las fosas nasales mediante el empleo de un espectrómetro de masas y proporcionan un perfil de masas del aroma liberado a tiempo real durante el período que dura el consumo del alimento. Además, la aplicación de esta metodología permite comprobar los compuestos que se liberan después del consumo y que pueden estar relacionados con la persistencia del aroma. La mayor parte de los estudios de liberación de aroma *in vivo* se han llevado a cabo con productos sólidos y semisólidos como geles, productos lácteos, gomas de mascar (Boelrijk y col., 2006; Brauss y col., 1998; Brauss y col., 1999; Linforth y col., 1999). Sin embargo, solo unos pocos estudios se han centrado en alimentos líquidos (Linforth y Taylor, 2000, Doyen y col., 2001; Linforth y col., 2002; Lasekan y col., 2009).

En el sistema de APCI-MS una interfaz dirige una fracción del aire exhalado a la fuente de ionización del espectrómetro de masas mediante el efecto Venturi. Aquí, los

volátiles son ionizados e introducidos en la región de alto vacío al espectrómetro de masas. Esta técnica se ha utilizado en un gran número de aplicaciones (Linforth y Taylor., 2000; Ruijschop y col., 2009; Linforth y col., 2010; Blee y col., 2011; Clark y col., 2011, etc.) y actualmente se comercializa con el nombre de MS Nose™ (Micromass-Manchester, UK) (**Figura 6**).

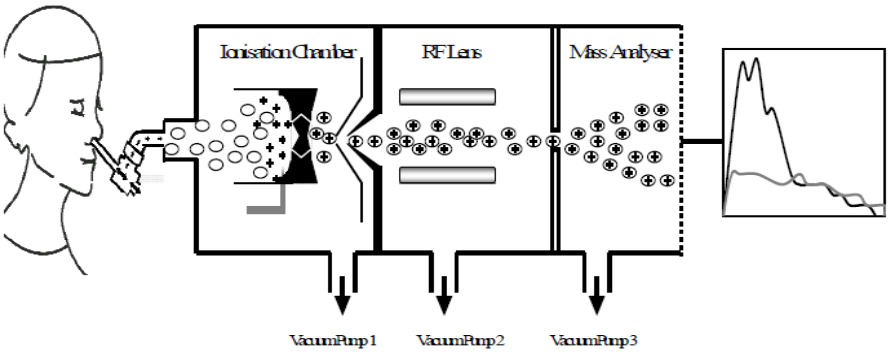


Figura 6: Diagrama esquemático del análisis mediante MS Nose™ (Fuente: Hollowood 2002, tesis).

Las principales características de la técnica de PTR-MS han sido revisadas en artículos previos (Lindinger y col., 1998; Hansel y col., 1998) y, como en APCI-MS se trata de una técnica muy sensible (**Figura 7**). En PTR-MS los compuestos volátiles son introducidos gracias al vacío del MS en la fuente de ionización. Los iones son extraídos y transferidos a la cabina de reacción (drift tube) donde tiene lugar la ionización química a temperatura y presión controlada, por lo que todos los componentes de la mezcla se ionizan en el mismo grado.

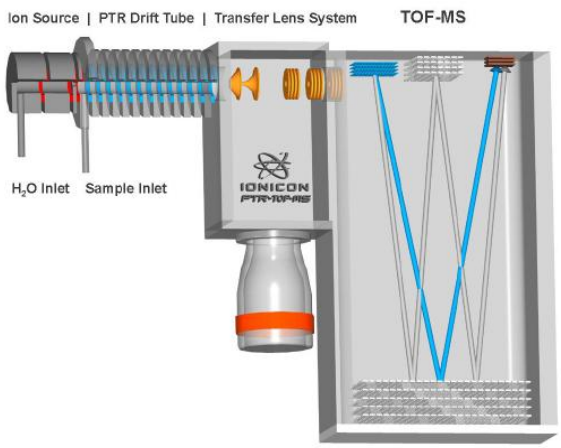


Figura 7. Esquema PTR-ToF-MS comercializado por Ionicon (http://www.americanlaboratory.com/913-Technical-Articles/34448-Technical-Advances-in-Proton-Transfer-Reaction-Mass-Spectrometry-and-New-Fields-of-Application/).

En ambas técnicas el ion primario usado para la ionización química es habitualmente el hidronio (H_3O^+) que se produce en la fuente de ionización. La ionización ocurre cuando se transfiere la carga del agua (H_3O^+) al analito. Los volátiles son detectados de acuerdo a su *m/z* ratio como masas correspondientes al ion molecular protonado (MH^+). En APCI-MS esto sucede a presión atmosférica mientras que en PTR-MS esto ocurre bajo vacío. Recientemente, en un estudio de comparación de las dos técnicas se ha comprobado que la APCI-MS presenta un límite de detección 10 veces menor y un rango lineal diez veces mayor que la PTR-MS (Avison 2013). Sin embargo, los resultados de otro estudio de comparación de ambas técnicas no han mostraron diferencias entre ellas (Deleris y col., 2013). Además, la PTR se ha combinado con otros tipos de analizadores diferentes al cuadrupolo, tales como la trampa iones (Warneke y col., 2004; Warneke y col, 2005) o el de tiempo de vuelo (Blake y col., 2004; Soukoulis y col., 2013; Tsevdou y col., 2013), lo que proporciona mayor sensibilidad y resolución (Heenan y col., 2012) permitiendo realizar los estudios de liberación de aroma en alimentos complejos, como el vino.

Ambas técnicas proporcionan perfiles de liberación tiempo-intensidad de los iones de interés bien resueltas, de tal manera que se pueden calcular algunos parámetros, como la cantidad total de los odorantes detectados en un cierto tiempo, y que es equivalente al área bajo la curva (AUC), la intensidad máxima de aroma (I_{max}), y el tiempo necesario para alcanzar la máxima intensidad (T_{max}) (**Figura 8**).

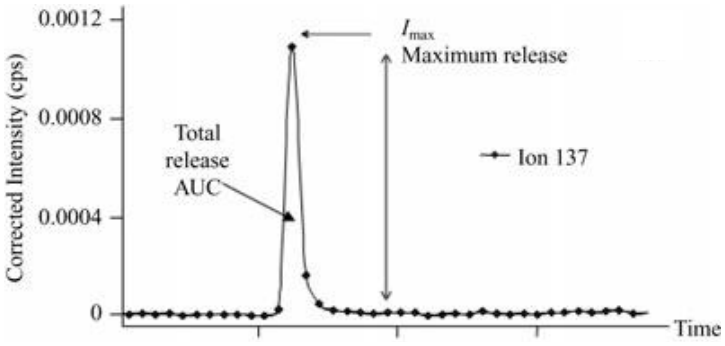


Figura 8. Curva de liberación del limoneno obtenida usando una garganta artificial acoplada a una PTR-MS (Fuente: Pozo-Bayón y col., 2009b)

Las ventajas más importantes del empleo de espectrómetros de masas para monitorizar el aroma en tiempo real, están normalmente relacionadas con tiempos cortos de respuesta (generalmente 200 ms o menos) y la relativamente alta sensibilidad de la técnica. Además el hecho de ser técnicas de ionización suaves, minimiza la fragmentación de los compuestos lo que implica una interpretación más sencilla de los resultados. Desafortunadamente, estas metodologías también presentan limitaciones, como son las relativas a la discriminación de algunos compuestos en el detector del instrumento, la presencia de interferencias procedentes de la matriz del alimento, la dificultad en la identificación de compuestos que presentan el mismo peso molecular (compuestos isobáricos), etc. Por otra parte, la falta de una separación previa a la detección del analito implica la superposición del espectro de todos los compuestos (fingerprint), que llegan al mismo tiempo al detector. Esto hace que dependiendo de la complejidad de la muestra analizada, la identificación del compuestos (y cuantificación) puede a veces ser difícil o imposible (Spitaler y col., 2007). Además, el análisis de los datos y la interpretación de los resultados requiere mucho tiempo y en ocasiones es problemática (Gierczynski y col., 2011). Otra de las desventajas que se han indicado recientemente es su dificultad para el estudio de aromas complejos, presentes en la mayoría de los alimentos, debido precisamente a la falta de selectividad y a la dificultad de interpretación de los espectros (Poinot y col., 2013).

A pesar del número creciente de aplicaciones de las técnicas basadas en PTR-MS y APCI-MS para monitorizar la liberación del aroma en tiempo real, estas técnicas apenas se han empleado en el caso del vino. Esto puede ser debido a algunos problemas relacionados con la composición de la matriz vínica, en concreto con el impacto del etanol en el proceso de ionización (Spitaler y col., 2007). Se ha comprobado que cuando el etanol está presente en una concentración superior a 100 $ppm_{v,}$ los iones primarios H_3O^+ pueden reaccionar con él para formar monómeros, dímeros, trímeros, aductos con moléculas de agua, iones fragmentados e incluso agrupamientos de etanol, que podrían reaccionar con los compuestos volátiles (Spitaler y col., 2007), de tal manera que sería imposible poder comparar muestras con diferente contenido en etanol. Se han propuesto dos posibles soluciones para evitar estos problemas. Una consiste en diluir el espacio de cabeza de la muestra con un flujo de nitrógeno saturado en etanol, desplazando los iones H_3O^+ por etanol protonado (Boscaini y col., 2004; Aznar y col., 2004). Otra solución propuesta en la literatura consiste en mantener los iones H_3O^+ como iones reactivos de

ionización química pero aplicando una dilución 40*x* al espacio de cabeza del vino con N_2 puro (Spitaler y col., 2007). Más recientemente, Fiches y colaboradores (2013) han propuesto un sencillo método basado en el control del proceso de ionización en el que la variación de la energía media de colisión en el "drift tube" durante el análisis de soluciones hidroalcohólicas (en un rango comprendido entre el 10-40 % (v/v)) permite realizar un análisis cuantitativo para caracterizar brandies en función de su grado de envejecimiento (Fiches y col., 2014). Sin embargo, todas estas aplicaciones han sido con fines dirigidos a la clasificación de muestras de distintas características y no para evaluación de liberación del aroma en tiempo real. Tan sólo en el trabajo de Deleris y col., 2011 se investigó la influencia de la deglución en la liberación de aroma durante el consumo de vodka empleando la técnica de PTR-MS.

1.2.1.3 Otros sistemas para la monitorización de aroma durante el consumo

1.2.1.3.1 Sensores

Diferentes sensores basados en la nariz electrónica, boca electrónica y lengua electrónica pueden utilizarse para la clasificación de productos alimenticios. En concreto, la nariz electrónica se basa en un conjunto de sensores que monitorizan compuestos volátiles presentes en un alimento. Sin embargo, el empleo de sensores no permite la identificación o caracterización de compuestos volátiles individuales ni tampoco la distinción entre moléculas odorantes o no, ya que los sensores no son comparables con el sistema de olfacción humano (Linforth y Taylor, 2000). Sin embargo, estas técnicas en combinación con el empleo de un panel sensorial se han empleado para predecir descriptores sensoriales en vinos tintos (Buratti y col., 2007).

1.2.1.3.2 Sistemas de modelización y predicción del aroma liberado

Con el fin de predecir liberación de aroma durante el consumo de alimentos se han desarrollado diferentes modelos mecanísticos que permiten establecer las bases de la transferencia de masa y predecir así la dinámica de la liberación de aroma durante el consumo, permitiendo un considerable ahorro de tiempo y dinero al disminuir el trabajo experimental. Algunos de estos modelos fueron revisados recientemente por De Roos (2006).

El uso de estos modelos requiere un detallado conocimiento de los procesos físico-químicos y fisiológicos que ocurren durante el consumo. Los casos más simples son los aplicados a muestras líquidas. En este caso, las primeras ecuaciones de liberación sólo tuvieron en cuenta el efecto de la dilución con la saliva (De Roos y Wolswinkel, 1994). Más recientemente, se han descrito nuevos modelos matemáticos que consideran la fisiología de la respiración y la deglución (Normand y col., 2004), o incluso la tasa de flujo salival y la presencia de mucosa oral (Rabe y col. 2004).

Es importante subrayar que estos enfoques necesitan ser confrontados con datos experimentales. Pese a que hasta el momento los modelos no han logrado incorporar correctamente toda la fisiología del proceso de consumo y se han enfocado más sobre el producto que sobre el procesado oral, la modelización es una técnica prometedora que va, probablemente, a experimentar un gran desarrollo en los próximos años.

1.3 Factores que pueden influir la liberación del aroma retronasal en condiciones de consumo

La composición de la matriz no volátil del vino podría afectar la liberación de aroma durante el consumo o aroma retronasal ya que podría modificar la partición de compuestos volátiles entre la matriz alimentaria y la cavidad oral. Por otra parte, la propia fisiología del individuo (flujos respiratorios, composición saliva, etc.) podría también condicionar el tipo y la cantidad de compuestos que van a interaccionar con los órganos olfativos e influir en última instancia la percepción del aroma. A continuación se detalla la información científica disponible relativa al efecto de ambos factores en la liberación de aroma durante el consumo.

1.3.1 Interacciones entre la matriz no volátil del vino y compuestos del aroma

Anteriormente se ha mencionado que los compuestos del aroma del vino pueden interaccionar a nivel cognitivo, provocando fenómenos de aditividad, sinergismo o antagonismo (Atanasova y col., 2005; Roudnitzky y col., 2011). Aparte de este mecanismo más relacionado con el procesamiento de estímulos sensoriales a nivel cerebral, los compuestos del aroma pueden interaccionar físicamente con los componentes no volátiles presentes en el vino (polifenoles, lípidos, proteínas, etc.),

modificando su distribución entre las fases líquida y gaseosa. Esta distribución está determinada por el coeficiente de partición (*K*), que puede determinar el grado de interacción de un compuesto volátil con la matriz no volátil y se expresa según la siguiente ecuación:

$$K_{al} = \frac{C_g}{C_l}$$

Donde K_{al} es el coeficiente de partición y C_g y C_l son las concentraciones de los compuestos volátiles en las fases gaseosa y líquida, respectivamente.

Para el estudio de las interacciones entre compuestos volátiles y la matriz no volátil del vino, las técnicas empleadas habitualmente han sido las del estudio del espacio de cabeza estáticas o en situación de equilibrio (ej, Ettre 1993; Voilley y col 1991; Whiton y Zoecklein, 2000; Escalona y col., 2001; Hartmann y col., 2002). Las técnicas de espacio de cabeza dinámico, aunque menos utilizadas representan mejor la situación dinámica de liberación del aroma que se produce durante el consumo (Langourieux y Crouzet, 1997; Dufour y Bayonove, 1999a,b). En las técnicas de espacio de cabeza dinámicas, además de los coeficientes de partición gas/líquido hay que considerar otros factores como el efecto de dilución o el del reequilibrado de la muestra, así como otros fenómenos de difusión o de convección.

Otros métodos para determinar interacciones como la diálisis en equilibrio (Lubbers y col., 1994) no implican medidas de la fase gaseosa. Asimismo, la naturaleza de una interacción puede determinarse mediante el empleo de métodos espectroscópicos, tales como la resonancia magnética nuclear (RMN) (Dufour y Bayonove, 1999; Jung y col., 2000). Además de las técnicas experimentales, cabe mencionar que se han desarrollado algunos modelos matemáticos, mediante los cuales se ha logrado predecir el efecto de la modificación de ingredientes específicos de una matriz en la retención y/o liberación del aroma en el alimento (Taylor 2002; Linforth y col., 1999; Tromelin y col., 2010). Sin embargo, en el caso del vino esta metodología no ha sido empleada.

Este tipo de interacciones entre compuestos del aroma y matriz no volátil del vino, va a producir diferentes efectos en el aroma que han sido estudiados en diferentes

trabajos científicos. La **Tabla 1** resume los principales efectos descritos en la bibliografía. El etanol, es uno de los principales componente del vino y se ha comprobado que puede modificar la polaridad de la solución alterando el coeficiente de partición de los compuestos volátiles entre la fase líquida (vino) y gaseosa (espacio de cabeza). Hay un consenso en considerar que la presencia de etanol en condiciones estáticas provoca un aumento de solubilidad de los compuestos volátiles reduciendo su presencia en el espacio de cabeza (Voilley y col., 1991; Conner 1998; Whiton y Zoecklein, 2000; Hartmann y col., 2002, Camara y col., 2006). Este efecto se puede traducir en la "supresión" de muchas de las notas aromáticas del vino, como se ha comprobado en soluciones vínicas modelo y vinos comerciales (Guth y col., 1997; Grosch 2001; Escudero y col., 2007). Aunque en la mayor parte de los estudios se ha observado un efecto general de retención de aroma, el grado de interacción es diferente en función de la concentración de etanol y del tipo de compuesto volátil ensayado. No obstante, hay que tener en cuenta que la mayoría de los estudios se han llevado a cabo en condiciones estáticas y sin considerar el aspecto dinámico que se produce durante el consumo.

Tabla 1. Interacciones entre compuestos de aroma y componentes de la matriz del vino descritos en la literatura.

Compuesto de la matriz	Efecto observado	Compuesto aromático	Aproximación analítica	Referencias
Etanol	Contribuye '*per se*' al aroma del vino Influye en la viscosidad del vino Enmascara/aumenta la percepción de algunos compuestos volátiles Actúa como un co-solvente con el agua, incrementando la solubilidad de los compuestos aromáticos (reducción del compuesto volátil en el espacio de cabeza) En dinámico mejora la transferencia de masa entre las fases líquida y gaseosa debido a diferencias en la tensión superficial entre el agua y el etanol (efecto Marangoni)	Alcoholes, ésteres, pirazinas, terpenos, cetonas, aldehídos, C13 norisoprenoides, ácidos	Análisis sensorial	Bayonove y col., 2000; Nurgel y Pickering, 2005; Zamora y col., 2006; Pineau y col., 2007; Escudero y col., 2007; Jones y col., 2008, Le Berre y col., 2007; King y col., 2013; Petrozziello y col., 2014
			GC-O	Guth, 1997; Grosch y col., 2001; Ferreira y col., 2002b; Villamor y col., 2013
			Técnicas de espacio de cabeza:	
			estáticas (HS-SPME, APCI-MS)	Voilley y col., 1991; Conner, 1998; Whiton y Zoecklein, 2000; Hartmann y col., 2002; Aznar y col., 2004; Athes y col., 2004; Camara y col., 2006; Le Berre y col., 2007; Robinson y col., 2009; Villamor y col., 2013; Petrozziello y col., 2014
			dinámicas (APCI-MS, Tenax trap)	Tsachaki y col., 2005; Aprea y col., 2007; Le Berre y col., 2007; Tsachaki y col., 2008; Tsachaki y col., 2009

Compuesto de la matriz	Efecto observado	Compuesto aromático	Aproximación analítica	Referencias
Glicerol	Contribuye al dulzor y a la viscosidad del vino No modifica la volatilidad relativa de los compuestos estudiados	Alcoholes, ésteres	Análisis sensorial	Lubbers y col., 2001; Nurgel y Pickering 2005; Jones y col., 2008
			Técnicas de espacio de cabeza: *estáticas* *(HS-SPME)*	Robinson y col., 2009
			dinámicas *(purge and trap)*	Lubbers y col., 2001
Compuestos fenólicos	Fuerte interacción con los compuestos de aroma, reduciendo o aumentando la volatilidad de los mismos (enlaces π-π o de tipo hidrofóbico). La mayoría de los estudios coinciden en que la magnitud de las interacciones es dependiente de la naturaleza estructural (polaridad y conformación espacial) tanto de los compuestos fenólicos como de los volátiles, y de la concentración ensayada	Aldehídos, pirazinas, ésteres, terpenos	Análisis sensorial	Aronson y Ebeler, 2004; Goldner y col., 2010; Lorrain y col., 2013;Petroziello y col., 2014
			GC-O	Villamor y col., 2013
			Técnicas de espacio de cabeza: *estáticas* *(HS-SPME, LC-SH)*	Escalona y col., 2001; Robinson y col., 2009; Goldner y col., 2010; Mitropoulou y col., 2011; Villamor y col., 2013; Petroziello y col., 2014
			dinámicas *(dilución exponencial, HS-SPME)*	Dufour y Bayonove, 1999; Jung y Ebeler, 2003; Aronson y Ebeler, 2004;
			RMN	Dufour y Bayonove, 1999; Jung y col., 2000

Compuesto de la matriz	Efecto observado	Compuesto aromático	Aproximación analítica	Referencias
Polisacáridos	Diferentes efectos en función de la naturaleza del compuesto de aroma, del tipo de polisacárido y de su concentración, así como del estado conformacional (condiciones de extracción y purificación). En general, las manoproteínas producen una disminución de la volatilidad de algunos compuestos de aroma por interacciones hidrofóbicas, que se traduce en una disminución de la intensidad de notas aromáticas	Ésteres, alcoholes, terpenos, ácidos, aldehídos, cetonas	Análisis sensorial	Comuzzo y col., 2006; Chalier y col., 2007; Jones y col, 2008
			GC-O	Comuzzo y col., 2006
			Técnicas de espacio de cabeza :	
			estáticas *(LC-SH,HS-SPME)*	Comuzzo y col., 2006; Chalier y col., 2007; Mitropoulou y col,, 2011
			dinámicas *(dilución exponencial, diálisis en equilibrio)*	Lubbers y col., 1994; Langorieux y Crouzet, 1997; Dufour y Bayonove, 1999, Chalier y col, 2007
			RMN	Dufour y Bayonove, 1999
Madera	Absorción de los compuestos de aroma a la lignina de la madera	Ésteres, aldehídos, terpenoides, alcoholes, pirazinas	Técnicas de espacio de cabeza estáticas	Escalona y col., 2001
			LLE	Ramírez-Ramírez y col., 2001
Otros componentes del vino	La adición de diferentes sales al vino provoca diferentes efectos en función de su concentración y de la naturaleza del compuesto	Alcoholes, ésteres, aldehídos	Técnicas de espacio de cabeza estáticas	Escalona y col., 2001

En condiciones dinámicas, es decir, empleando un gas inerte para diluir el equilibrio del espacio de cabeza, Tsachaki y col. (2005) mostraron que la presencia de etanol en un rango de concentraciones comprendido entre 90-230 mL/L ayudaba a mantener la concentración de volátiles en el espacio de cabeza. Este efecto fue el contrario al que se había encontrado en estudios previos realizados en condiciones estáticas (Aznar y col., 2004). Por tanto, los autores sugirieron que, en condiciones dinámicas el etanol mejora la transferencia de masa entre las fases líquida y gaseosa debido a diferencias en la tensión superficial entre el agua y el etanol. En soluciones hidroalcohólicas el etanol situado en la interfase aire/líquido se evapora creando un gradiente de tensión superficial (Li y col., 1993) que hace que las moléculas de etanol presentes en el líquido suban hacia la superficie para reemplazar a las que se van perdiendo. El movimiento de estas moléculas puede arrastrar consigo una gran cantidad de compuestos volátiles hacia las capas superficiales. Este efecto está causado por la denominada convección de Marangoni y fue probado por el mismo grupo de investigación unos años más tarde gracias al uso de imágenes térmicas (Tsachaki y col., 2008). La mayoría de los vinos presentan un contenido de etanol comprendido entre 100 y 145 ml/L, situándose por tanto en la zona donde bajo condiciones dinámicas, el etanol puede ayudar a mantener la concentración de volátiles en el espacio de cabeza (Tsachaki y col., 2005). Sin embargo, los mismos autores encontraron que el efecto Marangoni se ve cancelado por algunas proteínas y otras macromoléculas del vino, siendo perceptible tan sólo en disoluciones sintéticas o en vinos muy envejecidos (Tsachaki y col., 2009).

El glicerol es uno de los componentes más abundantes en el vino. Se produce principalmente durante la fermentación de glicerol-pirúvico al inicio de la fermentación alcohólica. Pese a que contribuye directamente al sabor del vino (Noble y Bursick, 1984) e imparte cierta viscosidad en el mismo (Nurgel y Pickering, 2005), se ha comprobado que no modifica la volatilidad relativa de compuestos del aroma (Lubbers y col., 2001).

Los polifenoles son un constituyente cuantitativamente muy importante de la composición no volátil del vino, sobre todo en vinos tintos. Juegan un papel decisivo en la calidad sensorial de los vinos, contribuyendo al gusto (amargor), sensación en boca (astringencia), y color de los mismos (Riberau-Gayon y col., 2000). Sin embargo, diferentes autores les han atribuido la capacidad de interaccionar con algunos

compuestos odorantes del vino, afectando su volatilidad y por tanto la percepción del aroma (Dufour y Bayonove, 1999a; Jung y col., 2000; Jung y col., 2003; Aronson y Ebeler, 2004; Lorrain y col., 2013). La mayoría de los estudios coinciden sin embargo, en que la magnitud de las interacciones es dependiente de la naturaleza estructural (polaridad, conformación espacial) tanto de los compuestos fenólicos como de los volátiles ensayados (**Tabla 1**).

El grupo de los flavonoides ha sido el más estudiado, y en general se ha comprobado que un aumento de la catequina (el compuesto monomérico más abundante en vinos tintos (120-390 mg/L)) produce una disminución en el espacio de cabeza de ésteres (acetato de isoamilo y hexanoato de etilo) y aldehídos (benzaldehído, hexanal) (Dufour y Bayonove, 1999a; Jung y Ebeler, 2003). Este efecto de retención de la catequina sobre ésteres se ha confirmado a nivel sensorial por Lorrain y col. (2013). Sin embargo, para otros compuestos (heptanona) se observó el efecto contrario o ningún efecto (acetato de isoamilo) (Jung y Ebeler, 2003). Además, recientemente se ha comprobado que los taninos (polifenoles poliméricos) procedentes de los hollejos de uvas pueden tener mayor influencia que los procedentes de las semillas debido a que el grado de polimerización en pieles es superior al de las pepitas (Mitroupoulu y col., 2011).

Además de la influencia de compuestos fenólicos de tipo flavonoideo, se ha comprobado que polifenoles no flavonoideos como el ácido gálico (muy abundante en vinos tintos (65-165 mg/L)), puede interaccionar con los compuestos volátiles debido a la presencia de enlaces π-π entre el enlace de hidrógeno del anillo galoil del compuesto fenólico y el anillo aromático del compuesto odorante (como pirazinas), y también a puentes de hidrógeno que ayudan a estabilizar la estructura. El impacto sensorial de esta interacción se ha demostrado para algunos compuestos como la 2-metilpirazina (Aronson y Ebeler, 2004), pero no ha sido relevante para otros (acetato de isoamilo, isobutirato de etilo, butirato de etilo y el octanoato de etilo) (Lorrain y col., 2013).

Los polisacáridos están presentes en el vino en un rango de 0.5 a 1.5 g/L (Will y col., 1991) y pueden provenir de diferentes orígenes, siendo los más habituales la pared celular de las uvas, la secreción o autolisis de levaduras usadas durante la fermentación alcohólica o debidos a la presencia de *Botrytis cinerea*, un hongo parásito de las viñas.

Esta diversidad de orígenes hace que se trate de un grupo de compuestos muy diverso en cuanto a composición y estructura. Los principales polisacáridos que podemos encontrar en un vino son arabinogalactanos derivados de uva (AGs) y arabinoproteínas (AGPs) que representan un 40 % del total. También podemos encontrar ramnogalacturanos II (RG-II), manoproteínas (MPs) y mananos. En general, la presencia de polisacáridos se asocia con notas positivas para la calidad del vino y por ello, se tiende a incrementar su contenido indirectamente (utilizando cepas de levadura productoras de manoproteínas o aplicando enzimas glucanasas) o directamente mediante la aplicación de preparados enológicos a base de levaduras inactivas ricos en estos compuestos (Pozo-Bayón y col., 2009b). Además, se les atribuye la capacidad de poder interaccionar con la fracción aromática del vino, aunque el efecto de estas interacciones es menos conocido y dependiente de la concentración, el tipo de polisacárido y de la naturaleza del compuesto volátil ensayado (Dufour y Bayonove, 1999b). Recientemente, Mitropoulou y col. (2011) indicaron la posible presencia de interacciones entre AGs y ciertos compuestos aromáticos que a concentraciones elevadas podrían formar agregados disminuyendo la accesibilidad de los compuestos volátiles a sus puntos de unión, como se ha puesto de manifiesto en otros estudios (Landy y col., 1995; Charlier y col. 2007).

Sin embargo, la dificultad que conlleva la purificación de los polisacáridos del vino implica que la mayoría de los estudios sobre interacciones de polisacáridos con compuestos de aroma se han llevado a cabo con manoproteínas secretadas al vino por levaduras durante la fermentación alcohólica o derivadas de la autolisis de levaduras durante el proceso de envejecimiento del vino sobre lías. Las manoproteínas son el segundo grupo más abundante de polisacáridos del vino (el 35 % del total) (Vidal y col., 2003). Se ha sugerido que las MP pueden retener compuestos de aroma y esta unión es de naturaleza hidrofóbica y dependiente tanto de la concentración de proteínas presentes en la glicoproteína como del tipo de compuesto aromático (Lubbers y col., 1994; Chalier y col., 2007). Además las condiciones de extracción y purificación de manoproteínas juegan un papel muy importante en el estado conformacional y su habilidad para interactuar con los compuestos del aroma (Langorieux y Crouzet, 1997; Chalier y col., 2007). Estos efectos de retención se han corroborado con estudios sensoriales que han confirmado la habilidad de MPs para disminuir la intensidad de las notas aromáticas del vino (Chalier y col., 2007). En otro estudio sobre interacciones de

aroma con derivados industriales de levadura (extractos de levadura y autolisados), Comuzzo y col. (2006) y Pozo-Bayón y col., 2009b, Pozo-Bayón y col., 2009c encontraron que estas macromoléculas modificaron fuertemente la composición del aroma del vino, aportando nuevos compuestos o modificando la volatilidad de los compuestos del aroma originalmente presentes en el vino. Desde un punto de vista sensorial, los autores apuntaron que la adición de extractos de levadura a vinos con fuerte carácter varietal es imperceptible pero en el caso de vinos no aromáticos puede aportar notas negativas en función de la dosis suministrada. Además, las MPs pueden también influir en el aroma del vino por fijación de precursores de aroma (como terpenos glicosilados) teniendo importantes consecuencias en el aroma varietal (Moio y col., 2004).

Las proteínas están presentes en vinos en un rango de concentraciones muy amplio (entre 30-269 mg/L) (Feuillat y col., 2000). Su concentración depende del tipo de uva y de la tecnología empleada durante su elaboración. Las proteínas del mosto y el vino tienen un peso molecular entre 25-35 kDa (Pueyo y col., 1993) y la mayoría son glicoproteínas. Las proteínas son excelentes agentes gelificantes, por lo que su presencia puede alterar la viscosidad de la solución. Además, gracias a la presencia de sus diferentes grupos químicos, ofrecen múltiples posibilidades de interacción química con muchas moléculas odorantes (Voilley y col., 2006). La mayoría de los trabajos centrados en el estudio del efecto de proteínas sobre compuestos del aroma se han realizado empleando manoproteínas, como se ha comentado anteriormente. Sin embargo, poco se ha reportado sobre el estudio de las interacciones con otras proteínas y compuestos del aroma. Hasta nuestro conocimiento el único estudio centrado en elucidar el efecto de proteínas (que no sean manoproteínas) en la liberación de aroma del vino fue el de Druaux y colaboradores (1995), en el que utilizaron un vino sintético y albúmina sérica bovina como proteína modelo. Se comprobó que esta proteína era capaz de unirse a la γ-decalactona, aunque su capacidad de unión fue mayor en un medio acuoso que en un vino modelo (pH 3.5 y 10 % etanol).

Otro tipo de interacciones que se ha comprobado pueden afectar a la composición aromática del vino es la retención de algunos compuesto aromáticos a sitios hidrófobos de la lignina (Ramírez-Ramírez y col., 2001; Chassagne y col., 2003; Ramírez-Ramírez y col., 2004) en el caso de vinos sometidos a envejecimiento en

madera, o la adsorción de aromas a las lías de levadura, que podría ser un mecanismo importante para la disminución del contenido de aroma total de los vinos (Chassagne y col., 2005). Aunque este tipo de interacciones podría tener menos relación con la composición del aroma retronasal durante el consumo, sí que serían de interés para explicar las diferencias de intensidad aromática en vinos sometidos a estas prácticas enológicas.

Sin embargo, es importante considerar que en la mayoría de los estudios sobre interacciones se ha estudiado el efecto de un componente específico de la matriz vínica sobre uno o varios compuestos volátiles empleando soluciones vínicas modelo. A pesar de la utilidad de estos estudios, hay que tener en cuenta que en el vino, este tipo de interacciones son muy complejas y sólo unos pocos trabajos han considerado la complejidad de la matriz vínica en su conjunto. Por ejemplo, Robinson y colaboradores (2009) llevaron a cabo un interesante estudio factorial para determinar el efecto de algunos de los componentes más importantes de la matriz (etanol, glucosa, glicerol, catequina y prolina) sobre 20 compuestos representativos del aroma del vino. Sus resultados corroboraron el importante efecto del etanol, seguido de la glucosa y un pequeño efecto de la catequina, glicerol y casi insignificante de la prolina. También Villamor y col. (2013) estudiaron el efecto combinado del etanol, concentración de taninos y fructosa en un vino modelo mediante HS-SPME-GC/MS.

En este sentido, la importancia del estudio del papel de la matriz no volátil del vino en la percepción del aroma se ha puesto de manifiesto en algunas investigaciones recientes. Pineau y colaboradores demostraron en el año 2007 que la β-damascenona presentaba un umbral de olfacción 1000 veces más alto en un vino tinto reconstituido que en una solución hidroalcohólica. Por ello, los autores sugirieron que los valores de OAV obtenidos para estos compuestos en soluciones hidroalcohólicas deberían ser revisados ya que podrían sobreestimar la contribución de este compuesto al aroma del vino. Otros dos estudios recientes han demostrado también el importante efecto que varios componentes de la matriz vínica (Jones y col., 2008) o la matriz en su conjunto (Sáenz-Navajas y col., 2010) ejercen en la percepción sensorial del aroma del vino.

Sin embargo, se puede concluir que a pesar del conocimiento adquirido sobre el papel de algunos componentes no volátiles del vino y su capacidad de interacción con moléculas específicas del aroma hay una falta de conocimiento sobre el efecto global

que ejerce la matriz vínica en la liberación de los componentes volátiles del vino, y prácticamente se desconoce el impacto que este parámetro podría ejercer en condiciones de consumo (aroma retronasal). Aunque el estudio de este tipo de interacciones no ha recibido tanta atención científica como otros aspectos relacionados con la caracterización del aroma del vino, es de gran interés considerarlas ya que podrían ser relevantes para comprender la percepción del aroma del vino.

1.3.2 Factores fisiológicos implicados en la liberación de aroma durante el consumo

Como se indicó en el **apartado 1.2** de la presente memoria cuando introducimos un alimento en la boca, se va a ver sometido a un conjunto de procesos interconectados entre sí, que a su vez producen una serie de cambios físicos y químicos en el alimento debido a la influencia de parámetros relacionados con la fisiología del individuo (presencia de saliva, composición mucosa, acción de la microbiota, flujos respiratorios, diferencias anatómicas de la cavidad oral, etc). Estos factores (junto con las propiedades de cada compuesto volátil y la composición de la matriz donde está contenido) pueden modificar la composición aromática inicial del alimento e influir en la liberación del aroma, modulando el tipo y la cantidad de compuesto que llegará a los receptores olfativos (Overbosch y col., 1991; Taylor, 2002; Salles y col., 2007). Pese a que está establecido que existen grandes diferencias interindividuales en la liberación de aroma durante el consumo, el origen de esta variabilidad no ha conseguido ser explicado completamente hasta la fecha. A continuación se van a repasar los principales factores fisiológicos descritos en la bibliografía que podrían influir la liberación del aroma durante el consumo de alimentos, y principalmente en el caso de alimentos líquidos como el vino.

1.3.2.1 Temperatura y pH orales

La temperatura corporal media se puede considerar de 37 ºC, siendo la media de temperatura típica oral de entre 36,8 ºC ± 0.4 ºC que puede considerarse estable o normoterma, es decir no se han encontrado grandes diferencias entre individuos. Sin embargo, la temperatura oral puede variar bruscamente durante el consumo de

diferentes alimentos (por ej., un helado *vs* una sopa) lo que puede modificar el reparto de los volátiles entre las fases líquida y gaseosa. De hecho, se ha demostrado que para un gran número de compuestos, una temperatura más elevada produce una mayor liberación de volátiles en la fase gaseosa (Roberts y Acree, 1995; Linforth y col., 2002) que puede ser debida a un aumento de los coeficientes de partición de los compuestos del aroma y a una disminución de la viscosidad de la matriz (Lubbers y Butler, 2010.) El mismo efecto se ha demostrado en situación de consumo *in vivo*, en la que se ha comprobado que un aumento de la temperatura de un alimento produce un incremento en la intensidad de aroma percibida (Ventanas y col., 2010; Engelen y col., 2003).

De igual manera, el pH de la cavidad oral es neutro (± 7) y estable no existiendo grandes diferencias entre individuos. Sin embargo, también puede sufrir grandes variaciones durante el consumo de alimentos (por ejemplo, vino *vs* leche).

Luego es importante tener en cuenta estas posibles variaciones de pH y temperatura durante la realización de estudios de liberación de aroma, ya que pueden alterar la estabilidad característica de la cavidad oral, modificando la cantidad de volátiles liberados.

1.3.2.2 Parámetros anatómicos

La anatomía de la cavidad oral de cada individuo puede determinar el modo en el que los alimentos pueden ser consumidos. Por ejemplo la geometría de la boca, nariz y garganta pueden condicionar los patrones de masticación y deglución, que se ha comprobado son dos procesos clave en la liberación de aroma durante el consumo (van Ruth y Roozen, 2000), lo que puede tener una gran influencia en la percepción del aroma (Mestres y col., 2006). Estudios recientes han mostrado grandes diferencias interindividuales en el modo en el que los alimentos son consumidos, mientras que el patrón de consumo parece ser consistente dentro de cada individuo (Kieser y col., 2011; Mishellany-Dutour y col., 2008; Ruijschop y col., 2009).

La masticación es el proceso clave en el caso de los alimentos sólidos y semisólidos, y es dependiente del estado dental (Mishellany-Dutour y col., 2008; Gierzcynski y col., 2011) y de la amplitud de los movimientos mandibulares (Pionnier y col., 2004a; Feron y col., 2014). En el caso de alimentos líquidos como el vino, el

tiempo de residencia del alimento en la boca es muy corto (2-3 segundos tras de la ingestión) y requieren un procesamiento mínimo en la cavidad oral por lo que pequeñas irregularidades en los patrones de consumo (diferencias en la deglución, profundidad de la respiración, movimientos de la mandíbula y la lengua, flujo de saliva, interacciones con las mucosas) pueden tener grandes consecuencias sobre la cantidad de aroma liberado (Boelrijk y col., 2006). Entre los parámetros anatómicos más relacionados con el consumo de alimentos líquidos destacan:

1.3.2.2.1 Lengua

La lengua es un órgano muscular móvil que juega un papel crucial en el manipulado, transporte y lubricación del alimento con la saliva en las diferentes fases del procesado oral. Además, los movimientos de la lengua afectan la percepción sensorial de textura, gusto y olor. Kieser y colaboradores (2011) encontraron diferencias interindividuales en la presión que ejerce la lengua durante la deglución. La importancia de la lengua en la liberación de aroma se ha evaluado en un reciente estudio por Benjamin y col. (2012) quienes diseñaron una boca artificial en la que entre otros parámetros (temperatura, flujo de saliva, flujos de aire, dimensiones orales), se tuvo en cuenta la presión que ejerce la lengua sobre la liberación de volátiles. Este dispositivo de boca artificial se controla desde un ordenador y se ha utilizado para evaluar la liberación de aroma en soluciones líquidas aromatizadas (Benjamin y col., 2012).

1.3.2.2.2 Barrera formada entre el velo del paladar y la lengua (barr-VL)

El velo del paladar forma una barrera con la lengua que impide y/o permite el paso de sustancias a la faringe. De hecho, la transferencia de aroma entre la cavidad bucal y nasal es dependiente de la posición del velo del paladar (Buettner y col., 2001), por lo que este es un factor crítico en la percepción de aroma durante el consumo.

Aunque en el caso de los alimentos sólidos, la barr-VL puede abrirse intermitentemente debido a los movimientos musculares vigorosos provocando una liberación continua de volátiles (Buettner y col., 2001; Hodgson y col., 2003), cuando un alimento líquido está presente en la boca, la barr-VL permanece cerrada y la cavidad

oral es un sistema estanco. Este segundo mecanismo conlleva una única etapa de transferencia de aroma que sucede tras la deglución y que se conoce como "swalow breath" (Land 1996; Buettner y col., 2001). Buettner y colaboradores (2008) aplicaron videofluroscopía y PTR-MS para visualizar este hecho durante el consumo de vino.

1.3.2.2.3 Volumen de aire en la cavidad oral (IMAC, In-Mouth Air Cavity)

La distancia de la lengua y el paladar influye en el volumen de aire presente en la cavidad oral, lo que puede condicionar la entrada de compuestos volátiles hacia los receptores olfativos (Buettner y col., 2002a). En una situación de reposo, la media de volumen de aire contenido en la boca es de 10 y 18 mL (Mishellany-Dutour y col., 2012), pero se han descrito grandes diferencias entre individuos (Bourdiol y col., 2013). Por otra parte, el volumen medio máximo de la cavidad oral durante el consumo se ha estimado en 40 mL, siendo 80 mL la capacidad total máxima (Linforth y Taylor, 2006; Poette y col., 2013).

Recientemente, en un interesante estudio llevado a cabo por Mishellany-Dutour y colaboradores (2012), se ha demostrado una correlación entre IMAC y aroma retronasal. Esta variable parece estar relacionada con la barrera formada entre el velo del paladar y la lengua. En este estudio, se seleccionó un grupo de personas que mostraron diferentes comportamientos durante el consumo de un caramelo de menta. El grupo de panelistas que liberaron mayor cantidad de aromas mostró unos valores de IMAC (medidos empleando un faringómetro acústico) pequeños y constantes lo que se relacionó con una posición de la lengua cercana al paladar y una apertura constante de la barr-VL. Sin embargo, los sujetos que presentaron unos valores de IMAC superiores (lo que se correspondió con una posición de la lengua más baja) liberaron menos cantidad de aroma. De hecho, estos autores sugieren que este grupo podía presentar el paladar más hueco de media que el del grupo de los mayores liberadores de aroma. Estos resultados están de acuerdo con los de un estudio anatómico llevado a cabo por Bourdiol y col., 2013., por lo que, aunque otros factores podrían estar influenciando este comportamiento, se estableció que el parámetro IMAC parece ser fundamental para explicar diferencias de liberación de aroma entre individuos durante el consumo.

1.3.2.3 Flujos respiratorios

Durante el consumo de alimentos, el aire exhalado sube de los pulmones, entra en la cavidad oral y transporta los compuestos volátiles hasta los receptores olfativos situados en la cavidad nasal. En este sentido, Voirol y Daget (1986) encontraron que la percepción de aroma estaba afectada por el volumen de aire que llega a los receptores olfativos. Por tanto, el estudio de los flujos respiratorios es esencial a la hora de entender el aroma liberado durante el consumo y debe ser implementado en sistemas *in vitro* para representar la situación dinámica que se produce durante el consumo de alimentos.

Aunque en general, se ha comprobado que una mayor capacidad respiratoria contribuye a arrastrar más volátiles hacia la cavidad nasal (Frank y col., 2011; Pionnier y col., 2004a), otro estudios en condiciones *in vivo* e *in vitro* observaron que un aumento del flujo respiratorio produce una disminución de la liberación de aroma por un efecto de dilución de aroma (Weel y col., 2004).

Una respiración irregular no varía sustancialmente el perfil de liberación durante el consumo de alimentos sólidos, ya que en general, el traspaso de aire es constante pero sí que puede tener un gran efecto durante el consumo de alimentos líquidos debido a que la liberación de aroma después de la deglución se traduce en un único pulso de aroma (swallow breath). Por ello, se ha sugerido que el establecimiento de protocolos de consumo es necesario para obtener datos reproducibles (Well y col., 2004; Aprea y col., 2007; Deleris y col., 2011; Salles y col, 2011) de liberación de aroma.

1.3.2.4 Saliva

La saliva es uno de los parámetros orofisiólogicos más estudiados por su importante implicación durante el procesado de los alimentos en su paso por la cavidad oral. De hecho, la presencia de la saliva es esencial durante el consumo de alimentos ya que permite la lubricación de la mucosa oral y ayuda a la formación del bolo alimentario (Prinz y Lucas, 1997). La saliva puede afectar al aroma retronasal por dilución, por interacciones entre compuestos de aroma y constituyentes de la saliva (como las proteínas), debido a su actividad enzimática o por su capacidad amortiguadora de pH (Spielman 1990; Odake y col., 1998). Recientemente se ha

sugerido que durante el consumo, no percibimos las propiedades intrínsecas de un alimento, sino la interacción resultante entre el alimento y la saliva (Neyraud 2014).

La saliva es un fluido biológico propio de la cavidad oral, secretado por tres glándulas principales (parótida, sublingual y submandibular), que producen aproximadamente el 90 % de la producción total, junto con cientos de glándulas salivares menores repartidas por la mucosa oral (Mese y Matsuo, 2007) (**Figura 9**). La saliva también contiene fluido del surco gingival, microorganismos de la placa dental y restos de alimentos (Pedersen y col., 2002).

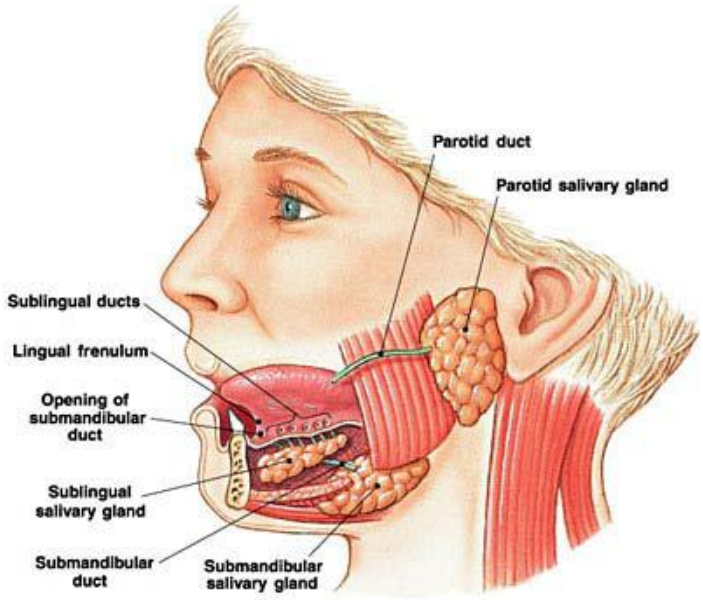


Figura 9: Representación esquemática de las diferentes glándulas salivares (http://entallergyandsinus.com/articles/the-throat/salivary-glands/).

La composición de la saliva es diferente en función de la glándula que la segregue y por ejemplo, las glándulas parótidas producen saliva más acuosa, con amilasa y proteínas ricas en prolina (PRP) (70 % de la proteína en la saliva parótida) (Mese y Matsuo, 2007) y las glándulas submandibular y sublingual producen saliva viscosa y rica en mucina (Mese y Matsuo, 2007). Las otras glándulas menores localizadas a lo largo de la mucosa oral producen pequeñas cantidades de saliva rica en mucina (Carpenter 2013). La saliva se compone principalmente de agua (99 %). El 1 % restante está constituido por moléculas inorgánicas y moléculas orgánicas, que son en su mayoría proteínas (1 a 3,5 mg/ml (Bennick 1982)). De hecho, se han identificado más

de un millar de proteínas en el proteoma salival (Denny y col., 2008), que está descrito como un sistema dinámico (Helmerhorst y Oppenheim, 2007) y complejo (Hirtz y col., 2005), debido a que la secreción de proteínas están controladas por numerosos factores genéticos, ambientales, etc. (Salles y col., 2011). El flujo de la saliva y la composición de la misma están sujetos a una alta variabilidad entre individuos (exposición a la luz, estimulación previa, circunstancias climatológicas, etc.) (Dawes 1981). Un par de estudios han comparado la variabilidad intra- e inter- individual en la composición de la saliva. Estos estudios encontraron que las variaciones intra-individuales son más pequeñas que las variaciones interindividuales (Jehmlich y col, 2013; Neyraud y col, 2012). Esto sugiere que la variabilidad interindividual (debido a polimorfismos genéticos o del medio ambiente, etc) debe tomarse en cuenta porque podría estar relacionada con otras variables como la percepción de alimentos y preferencias alimentarias.

La mayoría de los estudios hasta la fecha en relación con los efectos entre saliva, alimentos y liberación de aroma se han realizado utilizando simuladores *in vitro*, sobre varios tipos de alimentos: soluciones acuosas modelo (Roberts y Acree, 1995; Buettner y Schierberle, 2000; Friel y Taylor, 2001), soluciones modelo lipídicas (van Ruth y col, 2001), vegetales, como pimientos y judías (van Ruth y col., 1994, van Ruth y col., 1996, van Ruth y Roozen, 2000), alimentos complejos como ensaladas, (Odake y col., 1998) y vino (Genovese y col., 2009).

La composición de la saliva podría influir en la liberación de compuestos del aroma por interacción entre algunos de sus componentes, como las proteínas. Por ejemplo, se ha comprobado mediante la técnica de espacio de cabeza estático (Friel y Taylor, 2001; van Ruth y col., 2001) y por la técnica SOOM (Buettner y col., 2002a) que los aldehídos y otros compuestos del aroma pueden interaccionar con las proteínas de la saliva, especialmente con la mucina.

Algunos trabajos han estudiado la correlación entre el flujo de la saliva y la liberación de compuestos volátiles y varios de ellos han mostrado una reducción significativa en la liberación de aroma cuando aumenta el volumen de saliva (van Ruth y Roozen, 2000; Mehinagic y col., 2004; Haahr y col., 2004), de acuerdo con un modelo matemático propuesto por Harrison y col. (1998). En otro estudio, el aumento de saliva provocó un aumento de la volatilidad de algunos compuestos (Deibler y col., 2001;

Mitropoulou y col., 2011), pero no ejerció ningún efecto sobre otros (Deibler y col., 2001; Pionnier y col., 2004a,b). Incluso si no hay un consenso claro sobre el impacto del flujo de saliva en la liberación del aroma, varios estudios se han centrado en la correlación entre el flujo de saliva y la percepción temporal de compuestos volátiles y no volátiles. Noble (1995) encontró durante el consumo de vino, que los sujetos de bajo flujo salivar alcanzaron la intensidad máxima más tarde que los sujetos que presentaban un flujo alto y su percepción se alargaba en el tiempo. Del mismo modo, estudiando la percepción de la vainillina y limoneno en emulsiones de aceite/agua, Mialon y Ebeler (1997) mostraron que la tasa de flujo salival influye la percepción de aroma retronasal de vainillina pero no en la de limoneno. La diferencia observada entre los dos compuestos pudo ser debida al establecimiento de interacciones hidrofóbicas entre la vainillina y la saliva. En conclusión, se han demostrado efectos contradictorios de la tasa del flujo salival sobre la liberación de aroma, aunque la aplicación de metodologías diferentes no permite hacer comparaciones directas entre estudios. Además, otras variables implicadas en el proceso del consumo (como flujos respiratorios o el procesado en boca) no han sido tomadas en cuenta en los citados estudios.

Además en la saliva humana se han descrito unas 30 enzimas (que incluyen amilasa, invertasa, maltasa, anhidrasa carbónica, ureasa, oxidasa, catalasa, enzimas proteolíticas, lipasa, fosfatasa, lisozima, e hialuronidasa, entre otras). Esta diversidad de actividades enzimáticas sugiere una posible influencia de las enzimas en la percepción del sabor y aroma (Hussein y col., 1983; Buettner 2002b,c). Hussein y colaboradores (1983) señalaron la gran variabilidad entre sujetos en relación con la capacidad hidrolítica de la saliva. Durante los siguientes veinte años, sólo unos pocos estudios continuaron el trabajo de Hussein y col. (1983), y los debates sobre el tema fueron esencialmente teóricos. Recientemente, la acción enzimática de la saliva sobre compuestos de aroma ha sido demostrada en soluciones acuosas (Buettner, 2002a,b), o sugerida en alimentos, como el vino (Genovese y col., 2009), o “vino de palma” (Lasekan 2013). En concreto, se ha mostrado que los grupos químicos de ésteres, tioles y aldehídos son los grupos más implicados (Hussein y col., 1983 y Buettner 2002 b, c, Lasekan 2013). Sin embargo, en otros grupos químicos como las pirazinas y alcoholes no se ha observado un efecto metabólico (Lasekan 2013). Se ha sugerido que la degradación de ésteres puede ser debida a mecanismos de hidrólisis originados por la presencia de enzimas esterolíticas presentes en la saliva (Chauncey y col., 1954), y por

carboxilesterasas (Buettner 2002b; Hussein y col., 1983), sin embargo no hay datos concluyentes que demuestren este efecto. También las enzimas salivares podría intervenir en la degradación de aldehídos a ácidos carboxílicos por la NAD-alcohol deshidrogenasa o a la reducción a los correspondientes alcoholes por la NADP-aldehído reductasa y la de los tioles por la acción de la peroxidasa.

Sin embargo, varios autores han sugerido otras posibilidades que explicarían el efecto observado en algunos de los trabajos anteriormente citados. Por una parte, sugieren la posibilidad de que al inactivar la saliva (100 ºC, 10 min) para poder comparar los resultados entre saliva fresca y saliva sin enzimas, se puede producir una desnaturalización, agregación o precipitación de proteínas, o un cambio de las propiedades físicas de la saliva, en particular de la viscosidad de la misma, lo que también podría explicar los resultados obtenidos en la saliva inactiva (no degradación) (Lasekan 2013). Sin embargo, la posibilidad de interacciones moleculares entre la mucina con determinados compuestos volátiles no puede ser excluida. Es posible que la mucina de la saliva establezca enlaces hidrofóbicos con los compuestos de aroma (en concreto, de ésteres), causando una disminución en la concentración como se ha demostrado previamente por Friel y Taylor (2001). Más interesante es el hecho de que, de acuerdo con Friel y Taylor (2001), las sales presentes en la saliva podrían modificar el número de sitios de unión disponibles de la mucina lo que podría resultar en la formación de sitios de inclusión hidrofóbicos en la proteína que podrían atrapar compuestos volátiles, disminuyendo la cantidad de algunos tipos de compuestos volátiles (ésteres) en solución. Además, también se ha demostrado una interacción significativa entre la mucina de la saliva y aldehídos, a través de la formación de bases de Schiff (Friel y Taylor, 2001).

Por lo tanto, no se ha podido establecer ninguna conclusión general sobre el efecto que ejerce la saliva en la liberación de aroma debido a que su efecto varía en función de las condiciones experimentales empleadas. Principalmente, depende del compuesto de aroma estudiado (Buettner 2002b,c; Hansson y col., 2003; Boland y col., 2004), de la matriz en la que se encuentre (van Ruth y Roozen, 2000), de la composición de la saliva empleada en la realización del experimento (Friel y Taylor, 2001; Genovese y col., 2009; Poette y col., 2013), del ratio de dilución empleado (van

Ruth 2001) o del tiempo de incubación (que puede ir de 1 min a 3 h de acuerdo con la bibliografía) (Doyennette y col., 2011).

1.3.2.5 Microbiota oral

La cavidad oral presenta unas condiciones de temperatura (37 ºC), humedad y pH (alrededor de 7) que la convierten en un hábitat muy adecuado para el crecimiento microbiano. La cavidad oral aloja un amplio rango de bacterias Gram-positivas y Gram-negativas, así como algunas levaduras, micoplasmas y protozoos (Marsh, 2009). Se estima que la cantidad de microorganismos presentes en la cavidad oral es de aproximadamente 10^{11} bacterias/g en la placa dental y 10^{8}-10^{9} bacterias/mL en la saliva, mientras que por ejemplo en el colon (el órgano donde se encuentra la concentración bacteriana más elevada del organismo) se puede llegar a 10^{11}-10^{12} bacterias/g.

Sin embargo, la cavidad oral no es un ambiente homogéneo, sino que está constituida por distintos microambientes (**Figura 10**), cada uno de ellos colonizado por su propia microbiota.

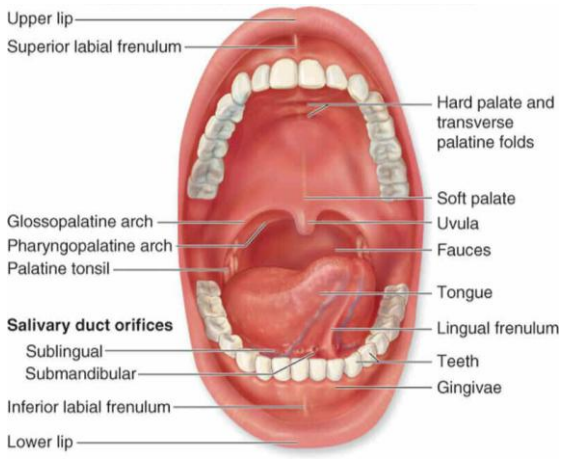


Figura 10. Microambientes de la cavidad oral humana (http://droualb.faculty.mjc.edu/Course%20Materials/Elementary%20Anatomy%20and%20Physiology%250/Lecture%20outlines/digestive_system%20alimentary%20canal.htm).

Por ejemplo, las superficies estables como los dientes, permiten la formación de biofilms complejos (placa dental) en especial en las regiones menos accesibles, como es el espacio entre los dientes y en los márgenes de las encías.

Las bacterias que predominan en la saliva proceden de las distintas superficies de la cavidad oral, especialmente de la lengua. Entre las especies bacterianas más comunes descritas en todos los microambientes de la boca se hallan los géneros *Gemella*, *Granulicatella*, *Streptococcus* y *Veionella* (Aas y col., 2005), aunque existe gran variabilidad interindividual atendiendo a factores como la dieta, la edad y la alimentación.

El desarrollo de la comunidad microbiana en la cavidad oral se inicia con la adhesión de especies de *Streptococcus, Actinomyces, Veillonella* y *Neisseria* (colonizadores primarios) a las superficies mucosas (Aas y col., 2005) (**Figura 11**). Dicha adhesión implica invariablemente la unión de las bacterias a los componentes de la saliva, que son adsorbidos a las superficies de la cavidad oral. Las condiciones anaerobias creadas por los colonizadores iniciales (early colonizers) promueven la incorporación de colonizadores secundarios (late colonizers), como *Fusobacterium spp*.

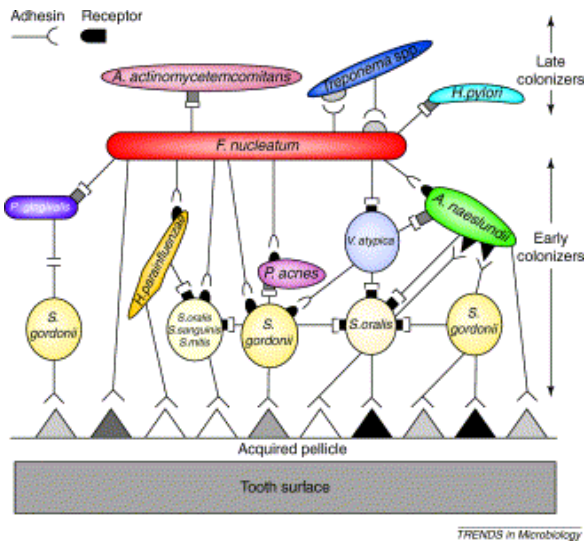


Figura 11. Representación esquemática de la naturaleza temporal de formación de un biofilm en la superficie dental (Fuente: Rickard y col., 2003).

Aunque los trabajos de la influencia de la microbiota oral en el aroma son prácticamente simbólicos algunos grupos de investigación han comprobado la capacidad de hidrólisis de compuestos fenólicos por la microbiota oral (Walle y col., 2005; Kamonpatana., 2012), sugiriendo que la boca es la primera etapa de metabolización para muchos componentes de los alimentos tras su ingestión. En el caso

de alimentos líquidos, como el vino, se ha sugerido que los volátiles presentes en solución pueden verse más afectados por este tipo de reacciones ya que no necesitan liberarse de la matriz sólida (Walle y col., 2005).

Starkenman y col., 2008, demostraron que algunas bacterias de la boca, en concreto las anaerobias pueden hidrolizar precursores no odorantes derivados de la cisteína presentes en algunos vegetales (uvas, cebollas, pimientos) liberando las correspondientes agliconas odorantes. En este estudio se evaluó la percepción retronasal tras el consumo de varios precursores derivados de la cisteína mediante análisis sensorial utilizando un ensayo de tiempo-intensidad. Los resultados del análisis sensorial demostraron claramente que en situación *in vivo* se produce esta hidrólisis en la boca que se traduce en una percepción de las correspondientes agliconas aromáticas a los 20-30 segundos después de ingestión, que se prolongó hasta 3 minutos después de la ingestión (persistencia de aroma). A pesar de observar un claro efecto en la percepción sensorial debido a la presencia de microorganismos, en este estudio no se pudo detectar la liberación de los tioles libres utilizando una técnica de análisis en tiempo real (APCI-MS). Esto pudo ser debido a que el límite de detección del instrumento era insuficiente para detectar estos compuestos, que se caracterizan por presentar muy bajos umbrales de detección. Por lo tanto, para probar el efecto de la microbiota bacteriana presente en la saliva, los autores incubaron los precursores en presencia de saliva en condiciones *in vitro* y monitorizaron la cinética de transformación de los precursores mediante la disminución de precursores por cromatografía líquida (LC-MS). Esta disminución del precursor en la saliva fue del orden 20 % tras 2 horas de incubación y del 80 % tras 24 horas. Sin embargo, en saliva estéril (sin microorganismos) solo se observó una disminución del 15 % tras 4 días de incubación.

Recientemente, Mayr y col., 2014 también han observado la degradación *in vivo* de precursores no odorantes de compuestos fenólicos volátiles, que en vinos están asociados a aromas desagradables como “tostado”, “quemado” etc, sugiriendo que este efecto podría ser debido a la acción de la microbiota oral.

Sin embargo, la liberación de agliconas volátiles a partir de precursores de aroma en vino por parte de la microbiota de la cavidad oral, no se ha confirmado analíticamente hasta la fecha.

1.2.3.6 Mucosa oral

La cavidad oral humana se halla completamente tapizada por una capa de mucosa formada por epitelio y tejido conectivo subyacente que presenta funciones de protección, secreción y absorción. La estructura de este epitelio no es homogénea a lo largo de toda la superficie, pudiendo ser de varios tipos. La mucosa de revestimiento está formada por epitelio escamoso estratificado no queratinizado que recubre los tejidos blandos (cara interna de los labios, cara interna de las mejillas, piso de la boca, cara inferior de la lengua) y permite mejorar la flexibilidad (articulación de palabras). Las áreas asociadas con la masticación (mucosa masticatoria) como el paladar duro y la encía, están sujetos a fuerzas mecánicas y por lo tanto están compuestas por epitelio queratinizado, como la epidermis de la piel. Y por último, la mucosa especializada se encuentra en los dos tercios anteriores de la cara dorsal o superficie superior de la lengua. Se llama especializada porque en ella se encuentran los receptores de sabor. La lengua está cubierta por un epitelio especial unido firmemente al músculo de la lengua y es una combinación de epitelio queratinizado y no queratinizado (Nicolazzo y col., 2005).

Recientemente, se está investigando el papel de la saliva en la formación de una película en la superficie de la mucosa (mucosal pellicle) formada por dos capas (**Figura 12**), en la cual la cara más externa estaría recubierta por mucina tipo MUC5B (Macakova y col., 2011). Esta glicoproteína, de alto peso molecular (>1,000 kDa), asegura protección y lubricación a la cavidad oral (Slomiany y col., 1996; Inoue y col., 2008; Boze y col., 2010) y podría jugar un papel importante en la percepción sensorial, por su efecto de retención de moléculas odorantes o potencialmente odorantes (Carpenter, 2013).

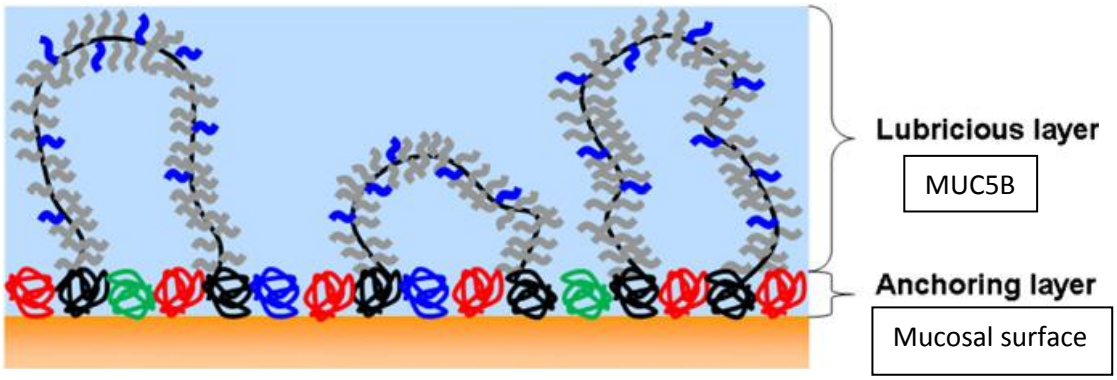


Figura 12. Estructura de la capa salivar (Fuente: Macakova y col., 2011)

Como se ha descrito previamente (**apartado 1.2**), tras la deglución se forma una fina capa del alimento en la boca y la faringe que puede ser un reservorio de moléculas de aroma listos para ser liberados de forma inmediata tras la primera exhalación tras el consumo, o de forma prolongada en el tiempo (Buettner y col., 2001; Buettner y col., 2002; Buettner y col., 2008). Para que esto suceda, los compuestos de aroma tienen que ser adsorbidos a la mucosa oral y/o faríngea y liberarse gradualmente provocando la sensación conocida como persistencia de aroma o "afterodor".

La persistencia de aroma debida a la adsorción de los compuestos de aroma en la mucosa oral se puede monitorizar *in vivo* mediante el empleo de sistemas de atrapamiento o técnicas espectrométricas (PTR-MS) y se ha comprobado en soluciones acuosas (Buettner y col., 2002a; Buettner y Welle, 2004), vino (Buettner 2004) y vino de palma (Lasekan 2013). Sin embargo, en un dispositivo de boca artificial desarrollado por Rabe y col. (2004), la presencia de mucosa no ejerció influencia en la liberación de aroma. No obstante, en este estudio no se evaluó la capacidad de la misma en la persistencia de aroma. Por otra parte, Weel y colaboradores (2003) observaron que el grado de interacción entre los compuestos del aroma y la mucosa depende fuertemente del tiempo de contacto.

Recientemente Goodstein y col., 2014 han desarrollado un estudio sensorial tiempo-intensidad en el que se ha observado que las notas aromáticas frutales son menos persistentes que las de coco, setas o florales en vinos blanco modelo.

Por lo tanto, las interacciones entre componentes de los alimentos y las superficies mucosas de la cavidad oral pueden jugar un papel importante a la hora de determinar muchos de los atributos sensoriales de los alimentos como suavidad, astringencia, persistencia del aroma (Malone y col., 2003). La evaluación de la acción

de las mucosas en estudios de persistencia de aroma es clave para entender la sensación percibida tras el consumo de un alimento y puede jugar un papel muy importante asociado con las preferencias de los consumidores. Sin embargo, a pesar de la importancia de la persistencia del aroma para definir la calidad de los vinos, en la actualidad no hay ningún estudio encaminado a evaluar el papel de la mucosa oral y el efecto de la matriz no volátil del vino en la capacidad de retención y posterior liberación de compuestos aromáticos.

Justificación y objetivos

2. JUSTIFICACIÓN Y OBJETIVOS

Como se ha comentado con anterioridad, el aroma constituye uno de los factores más importantes que influyen en la calidad del vino y condiciona las preferencias de los consumidores. Por ello la identificación y cuantificación de los compuestos volátiles responsables del aroma mediante el empleo de técnicas analíticas avanzadas (GC-MS, GC x GC, GC-O) ha constituido y constituye la mayor parte de la investigación sobre el aroma del vino. Por otra parte, la aplicación de técnicas de análisis sensorial ha permitido comprender el significado sensorial de muchos de los compuestos del perfil volátil del vino, jerarquizar su importancia odorante e incluso reconstruir el aroma de algunos tipos de vino. En conjunto, el desarrollo de estas técnicas ha proporcionado un valioso *background* de conocimiento sobre los compuestos del aroma del vino, y teniendo en cuenta la magnitud de esta área, es probable que estos trabajos continúen aportando una inestimable información en el futuro.

Sin embargo, actualmente se reconoce que la identificación de los compuestos del aroma del vino es una pieza más del rompecabezas que intenta explicar cómo se produce la percepción del aroma durante el consumo y su relación con las preferencias de los consumidores. Nuevos estudios han indicado la necesidad de considerar la composición de la matriz no volátil del vino a la hora de evaluar la percepción del aroma, ya que puede modificar en gran medida el umbral de percepción asignado para un determinado compuesto odorante (Pineau y col., 2007).

También en trabajos recientes se ha comprobado que el aroma ortonasal y retronasal que se experimenta durante el consumo puede ser diferente (Burdach y col., 1984; Voriol y Daget, 1986; Kuo y col., 1993; Aubry y col., 1999). El desarrollo de nuevas técnicas de análisis de liberación de aroma durante el consumo está permitiendo entender cómo se libera el aroma de los alimentos y su transporte a los órganos olfativos. Además, este tipo de metodologías en combinación con técnicas de tipo médico-analíticas (resonancia magnética de imagen en tiempo real, fMEI), han permitido confirmar la importancia de muchos procesos fisiológicos en la liberación de aroma que tienen lugar durante el consumo. A pesar de que en el caso de alimentos líquidos, como el vino, el procesamiento en la cavidad bucal es menor comparado con

los alimentos sólidos, hay importantes factores fisiológicos como la deglución, los flujos de aire, la interacción con la saliva, la presencia de microorganismos o la interacción con las mucosas que pueden condicionar la composición del aroma retronasal durante el consumo y por tanto el tipo de aroma disponible para interaccionar con los órganos olfativos. A pesar de la gran relevancia del aroma en la calidad de los vinos, este tipo de estudios en los que se considera la fisiología oral como mecanismo modulador del aroma retronasal durante el consumo son prácticamente inexistentes.

En base a lo expuesto, la **hipótesis de partida** del presente trabajo es que la matriz no volátil del vino y algunos parámetros relacionados con la fisiología oral pueden influir en la liberación de aroma en condiciones de consumo, afectando la cantidad y composición del aroma retronasal, y en última instancia la percepción del aroma del vino.

A partir de esta hipótesis, el **objetivo** de la presente Tesis Doctoral ha sido estudiar de manera conjunta, mediante el empleo de modelos de simulación de aroma retronasal *in vitro* así como en condiciones *in vivo*, cómo se produce la liberación del aroma en condiciones de consumo del vino, qué factores orofisiológicos (saliva, microbiota oral y mucosas orales) están implicados en este proceso, y cómo éste puede ser modulado dependiendo de la composición de la matriz vínica. El tema planteado es novedoso desde un punto de vista científico y de gran interés para la industria enológica centrada principalmente en la elaboración de vinos de alta calidad y dirigidos a grupos de consumidores específicos (consumidores target). Los resultados que se deriven de este trabajo contribuirán a explicar los fenómenos que influyen en la liberación y percepción del aroma del vino.

Los objetivos concretos que se persiguen son:

1- Estudiar el efecto de la matriz del vino en la capacidad de retención de compuestos de aroma del vino (**Apartado 4.1**).

2- Evaluar el efecto de la matriz del vino en la liberación del aroma retronasal en condiciones de consumo (**Apartado 4.2**).

3- Evaluar el impacto de parámetros relacionados con la fisiología oral (saliva, microbiota de la cavidad de oral y mucosas orales) en el aroma retronasal.

a. Evaluar la influencia de la saliva considerando el efecto de la matriz del vino en la liberación del aroma retronasal (**Apartado 4.3.1**).

b. Evaluar el papel de la mucosas oral en la retención y posterior liberación de compuestos del aroma del vino (**Apartado 4.3.2**).

c. Estudiar el efecto de la microbiota bacteriana de la cavidad oral en la generación de compuestos odorantes a partir de precursores no odorantes de la uva (**Apartado 4.3.3**).

El presente trabajo de investigación ha sido realizado gracias a la financiación del Ministerio de Economía y Competitividad (MINECO) a través del Proyecto de Investigación AGL2012--04172-C02-01 y a la concesión de una Beca JAE Predoctoral del CSIC (2010-2014).

Plan de trabajo

3. PLAN DE TRABAJO

Para la consecución de los objetivos planteados en la presente Tesis Doctoral, se siguió el siguiente plan de trabajo, presentado de forma esquemática en la **Figura 13**.

1) Estudiar el efecto de la matriz del vino en la capacidad de retención de compuestos de aroma del vino en condiciones estáticas:

In vitro HS-SPME-GC/MS

2) Evaluar el efecto de la matriz del vino en la liberación del aroma retronasal en condiciones de consumo (dinámicas):

In vitro Boca artificial-PTR-ToF-MS
In vivo Sistema de atrapamiento de aroma retronasal-GC/MS

3) Evaluar el impacto de parámetros relacionados con la fisiología oral (saliva, microbiota de la cavidad de oral y mucosas orales) en el aroma retronasal:

a) Influencia de la saliva considerando el efecto de la matriz del vino en la liberación del aroma retronasal, empleando condiciones estáticas y dinámicas:

In vitro HS-SPME-GC/MS

b) Influencia de la mucosa oral en la retención de compuestos del aroma del vino y su posterior liberación:

In vivo SOOM-GC/MS e Intraoral HS-SPME-GC/MS

c) Papel de la microbiota oral en la generación de compuestos odorantes a partir de precursores aromáticos de la uva:

In vitro y ex vivo HS-SPME-GC/MS

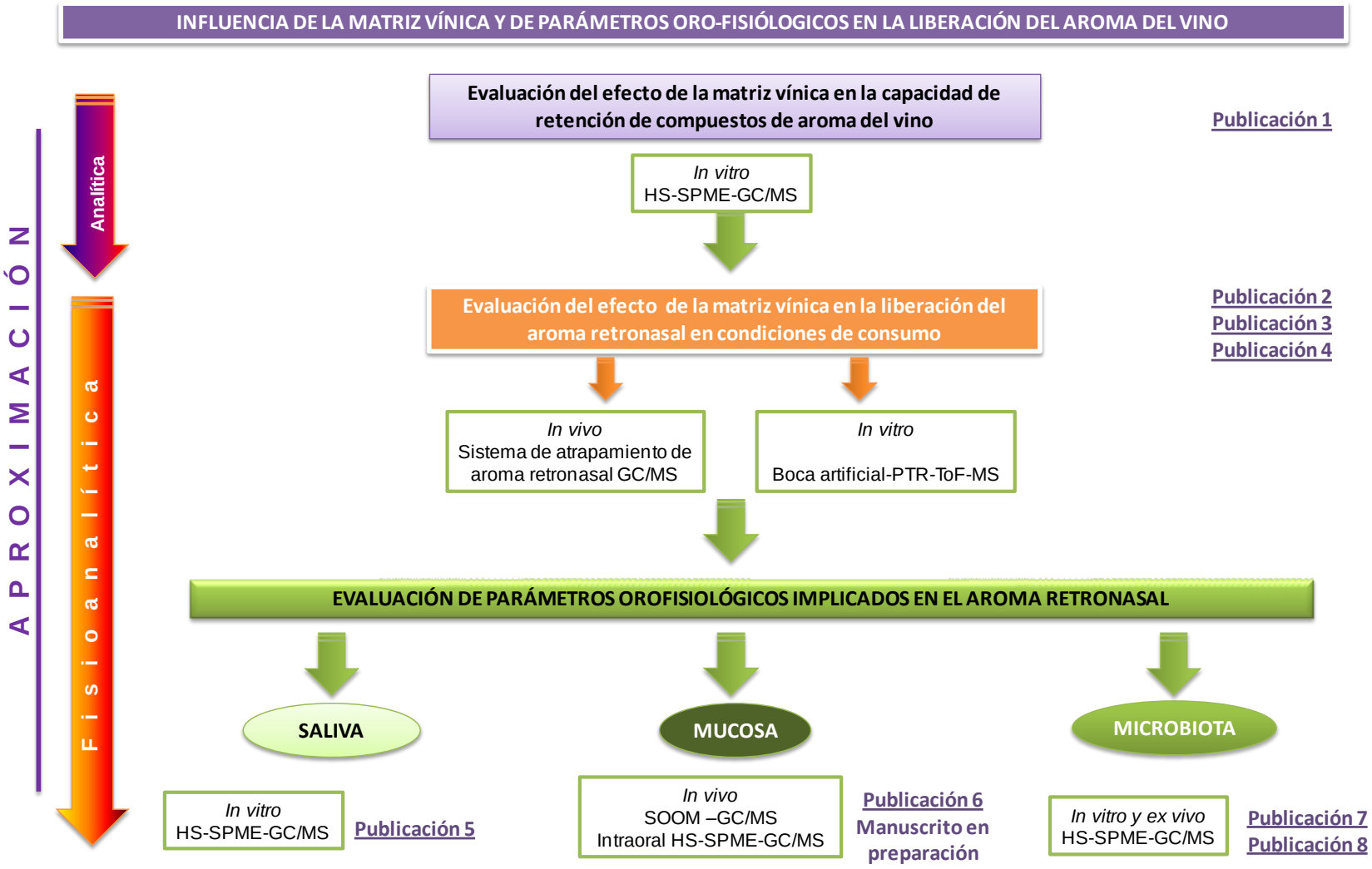


Figura 13. Esquema general del plan de trabajo llevado a cabo en la presente memoria.

Resultados

4. RESULTADOS

En esta sección se exponen los resultados obtenidos durante la presente Tesis Doctoral en base a la hipótesis y objetivos propuestos. Estos resultados se recogen en 8 publicaciones científicas (4 publicadas, 3 enviadas y 1 en preparación).

Además, durante la realización de la presente Tesis Doctoral se ha realizado un artículo de revisión (**Carolina Muñoz-González**, Juan J. Rodríguez-Bencomo, M. Victoria-Arribas, M. Ángeles Pozo-Bayón. "Beyond the characterization of wine aroma compounds: looking for analytical approaches in trying to understand aroma perception during wine consumption". *Analytical and Bioanalytical Chemistry*, 401 (5), **2011**, 1501-1516), que recoge los principales avances en el análisis del aroma del vino considerando las técnicas más innovadoras en el estudio de la liberación del aroma en condiciones de consumo.

Por último, los resultados derivados de esta Tesis Doctoral han sido seleccionados para ser presentados en forma de comunicación oral bajo el título "Oral physiological factors might be an important piece of the puzzle to explain aroma perception during wine drinking" en el 14th Weurman Flavour Research Symposium, Cambridge, United Kingdom, 15-19-Sept-2014. **Carolina Muñoz-González**, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón.

4. 1 Efecto de la matriz del vino en la capacidad de retención de compuestos de aroma del vino.

Como se describe en la sección de **Antecedentes bibliográficos**, la mayor parte de los esfuerzos encaminados a explicar el aroma de los vinos se han centrado en la caracterización de los compuestos volátiles responsables de su olor, y hoy en día se sabe que está integrado por más de mil compuestos (Polaskova y col., 2008). Sin embargo, numerosos estudios en la bibliografía han mostrado que algunos de los componentes no volátiles del vino (polisacáridos, polifenoles, etc) pueden interaccionar con los compuestos del aroma, afectando su volatilidad, y en último término, la percepción del aroma por parte del consumidor (Jones y col., 2008; Sáenz-Navajas y col., 2010). No obstante, la mayoría de los estudios analíticos encaminados a determinar la naturaleza de estas interacciones, se han llevado a cabo utilizando agua o soluciones hidroalcohólicas, que contienen un número limitado de macromoléculas y que se aromatizan con unos pocos compuestos volátiles representativos del aroma del vino. Los resultados de estos estudios tienen gran valor para determinar la existencia de interacciones pero son difícilmente extrapolables a la situación real que se produce en los vinos debido a su gran complejidad composicional y variedad de compuestos volátiles que contienen.

Era importante, por tanto, plantear un estudio sistemático que permitiera evaluar el efecto del conjunto de la matriz vínica sobre compuestos representativos del aroma del vino. Estas premisas nos llevaron a la selección de cinco vinos comerciales (blanco, espumoso, dulce, tinto joven y tinto crianza) con una composición química muy diferente. Los vinos se liofilizaron, desaromatizaron y reconstituyeron a la misma concentración de etanol (12 % v/v). Todos ellos se suplementaron con concentraciones crecientes de una mezcla formada por 36 compuestos del aroma del vino de las principales familias químicas (ésteres, alcoholes, terpenos, C13-norisoprenoides, fenoles volátiles, compuestos bencénicos, lactonas, compuestos furánicos y ácidos). Los compuestos del aroma se evaluaron en el espacio de cabeza de los vinos por HS-SPME-GC/MS. Las líneas de regresión obtenidas para cada matriz vínica se compararon con las obtenidas en un vino control aromatizado (sin efecto matriz), para de esta forma evaluar el efecto de la matriz en la liberación del aroma al espacio de cabeza.

A continuación se presentan los resultados de este trabajo en forma de publicación científica:

Publicación 1. Juan J. Rodríguez-Bencomo, **Carolina Muñoz-González**, Inmaculada Andújar-Ortiz, Pedro J. Martín-Álvarez, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón. "Assessment of the effect of the non-volatile wine matrix on the volatility of typical wine aroma compounds by headspace solid phase microextraction/gas chromatography analysis". *Journal of the Science of Food and Agriculture*, 91 (13), **2011**, 2484-2494.

- Además este trabajo fue presentado como comunicación oral titulada "Assessment of the effect of non-volatile wine matrix on the volatility of typical wine aroma compounds by HS-SPME-GC-MS analysis" en el 28th International Symposium on Chromatography, Valencia, España, 12-16-Sept-2010. Juan J. Rodríguez-Bencomo, **Carolina Muñoz-González**, Inmaculada Andújar-Ortiz, Pedro J. Martín-Álvarez, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón.

Publicación 1. Evaluación del efecto de la composición de la matriz no volátil del vino en la volatilidad de compuestos típicos del aroma empleando microextracción en fase sólida y análisis por cromatografía de gases.

Assessment of the effect of the non-volatile wine matrix on the volatility of typical wine aroma compounds by headspace solid phase microextraction/gas chromatography analysis

Juanjo J. Rodríguez-Bencomo, **Carolina Muñoz-González**, Inmaculada Andújar-Ortiz, Pedro J. Martín-Álvarez, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón

Journal of the Science of Food and Agriculture, 91 (13), 2011, 2484-2494

Abstract

BACKGROUND: The aim of this study was to evaluate the effect of the whole non-volatile wine matrix composition on the volatility of typical wine aroma compounds by comparing the slopes of regression lines of five deodorized and reconstituted wines with the slopes calculated for the same compounds in a control wine with no matrix effect.

RESULTS: The main effect observed was a reduction in the slopes, or a retention effect, that was largest for the reconstituted sparkling wine, which showed between 11% and 69% lower slopes than the control wine. In addition, an increase in the slopes, or a "salting out" effect, in the most compositionally complex reconstituted aged-red and sweet wines was also noticed for some volatiles with very low boiling point or low hydrophobic constant value.

CONCLUSION: This study has shown that the non-volatile composition of wines strongly affects the volatility of wine aroma compounds. In addition, the aroma chemical class, in particular its physicochemical properties (volatility and hydrophobicity) strongly influence this behaviour. On the basis of these results, many odour threshold values calculated in simple hydroalcoholic solutions and usually employed to evaluate the odour importance of specific volatile compounds may have been over- or underestimated.

Introduction

Aroma is one of the main characteristics in defining the quality of wines. Therefore many works in the scientific literature have been devoted to the identification and quantification of the key aroma compounds responsible for specific aromatic nuances in wines (Campo et al., 2005; Escudero et al., 2007; Ferreira et al., 1998; Ferreira et al., 2002; Guth 1997; Kotseridis & Baumes, 2000). However, aroma perception of a wine is strongly influenced by the way indigenous aroma molecules are distributed between the gas and liquid phases, which is characterized by the partition coefficient. Partitioning of volatile substance between the liquid and gas phases is mainly governed by aroma compound volatility and solubility (Voilley 2006). These physicochemical properties are expected to be influenced by wine constituents present in the medium, such as polysaccharides, mono- and disaccharides, polyphenols and proteins among others (Pozo-Bayón & Reineccius, 2009). The interaction between aroma molecules and wine non-volatile compounds might influence aroma release and ultimately ortho- and retro-nasal aroma perception.

Many wine matrix non-volatile components (carbohydrates, protein and polyphenols) come from the skin and pulp of the grapes and from the cell wall of the fermentation yeast. In addition, ethanol, produced during wine fermentation, represents a mayor wine matrix component. The great importance of considering the wine matrix in the perception of some important wine aroma compounds has been evidenced recently by Pineau et al., 2007 who showed that the odour threshold of β-damascenone was over 1000-fold lower in hydroalcoholic solution than in a reconstituted red wine.

Some research has been devoted to studying the interactions between aroma compounds and specific wine matrix constituents. Dufuour and Bayonove (1999) confirmed the existence of hydrophobic interactions between catechins and some types of aroma compound, and in a more recent study it was shown that gallic acid (in 10 mL L^{-1} ethanol solution) significantly decreased the volatility of 2-methoxypyrazine, while naringine at the same level had little effect (Aronson & Ebeler, 2004).

The effect of wine polysaccharides, mainly those derived from yeast cell walls, such as mannoproteins, on the volatility of aroma compounds has also been proved (Langorieux & Crouzet, 1997; Lubbers et al., 1994). The extent of this effect depends on the type of mannoprotein and even on the yeast strain (Chalier et al., 2007).

Moreover, different effects of yeast macromolecules released by different types of inactive yeast preparation commonly used to enhance fermentations on the volatility of typical wine aroma compounds have been shown recently (Pozo-Bayón et al., 2009).

Ethanol, the main wine matrix component, has the capacity to modify the solution polarity, thus altering the gas/liquid partition coefficient. The effect of increasing amounts of ethanol in decreasing wine aroma volatility has been very well documented (Conner et al., 1998; Escalona et al., 1999; Robinson et al., 2009; Rodriguez-Bencomo et al., 2002; Whiton & Zoecklein, 2000).

However, most studies on the effect of wine matrix components on the volatility of aroma compounds have been carried out using artificial wine matrices, usually aqueous or hydroalcoholic solutions, containing a very limited number of wine components and spiked with several types of aroma compound. Although this can be a valuable approach to determine the role of some specific matrix components, the results can rarely be extrapolated to real wines because of the latter's greater compositional complexity and wider variety of volatile chemical classes. In an attempt to obtain more information on the effect of wine matrix composition on aroma volatility, Robinson et al., 2009 recently presented an interesting full factorial design to assess the matrix effects of ethanol, glucose, glycerol, proline and catechin on the volatility of 20 wine aroma compounds, in which they corroborated previous results related to the large effect of ethanol, followed by glucose, and the small effect of catechin, glycerol and proline.

However, the effect of the whole non-volatile composition from real wine matrices on representative wine volatile compounds has not been study so far. Therefore the objective of this work was to study the effect of five types of wine matrix representing a wide range of wine compositions, which were previously deodorized and reconstituted to the same ethanol concentration, on representative chemical groups of wine aroma compounds. To do this, a comparison of the regression lines obtained by headspace solid phase microextraction/gas chromatography/mass spectrometry (HS-SPME/GC/MS) in each reconstituted matrix was performed and the results are discussed based on the physicochemical characteristics of the aroma compounds and on the chemical composition determined in each wine matrix.

Material and methods

Wines Samples

Five commercial wine samples representative of different wine matrix compositions were selected for this study: a young Chardonnay white wine (wine 1), a young Beaujolais red wine (wine 2), an oak-aged Tempranillo red wine (wine 3), a Cava white wine (Spanish sparkling wine manufactured by the traditional method) (wine 4) and a sweet biologically aged wine made from Pedro Ximénez grapes (wine 5).

Reconstituted Wines

Deodorization procedure

The wines (120 mL of each) were deodorized by treating them in an ultrasound bath for 20 min, then adding 15 g of Amberlite XAD-2 (Supelco, Bellefonte, PA, USA) and stirring for 1h. The wines were subsequently filtered through glass wool. The whole procedure was repeated twice. This procedure allowed the complete elimination of all aroma compounds in the wines (confirmed by SPME-GC/MS analysis).

Wine Reconstitution

The deodorized wines (8 mL of each) were transferred to 20 mL vials (Agilent Technologies, Palo Alto, CA, USA) and completely dried in a lyophiliser (Labconco, Kansas City, MO, USA). A total of 60 samples were prepared using this procedure (12 per wine type). The dried wines were weighed to calculate the repeatability of the liophylisation process. The residue after lyophilisation was reconstituted with 120 mL L^{-1} hydroalocholic solution to a final volume of 8 mL and spiked with the volatile mixture at five different concentration levels (**Table 1**). Duplicates of each reconstituted wine were prepared by this procedure. Besides the five types of reconstituted wine matrix, a control wine representing a sample with “no matrix effect” was prepared by mixing 120 mL L^{-1} ethanol with 4 g L^{-1} tartaric acid (Panreac, Barcelona, Spain) and adjusting the pH to 3.5 with NaOH (Panreac).

Table 1. Retention time, ion of quantification (Q), physicochemical characteristics and range of concentration assayed for each volatile compound studied.

Compound	CAS number	Retention time (min)	Ion Q (*m/z*) [h]	Boiling point (ºC)	log *P* [i]	Concentration range (mg L^{-1})
Isobutyl acetate [a]	110-19-0	4.61	56	116.5	1.78	0 - 0.675
Ethyl butyrate [a]	105-54-4	5.19	71	121.5	1.85	0 - 1.456
Ethyl 2-methylbutyrate [a]	7452-79-1	5.64	57	133	2.26	0 - 0.803
Butyl acetate [b]	123-86-4	6.22	43	126.1	1.78	0 - 0.713
Isoamyl acetate [b]	123-92-2	7.78	70	142.5	2.25	0 - 1.619
Ethyl hexanoate [c]	123-66-0	11.54	88	167	2.83	0 - 2.356
Hexyl acetate [b]	142-92-7	13.08	56	171.5	2.83	0 - 2.394
1-Hexanol [a]	111-27-3	16.32	56	157.6	2.03	0 - 2.200
trans-3-Hexen-1-ol [a]	928-97-2	16.64	67	156.5	1.61	0 - 0.875
cis-3-Hexen-1-ol [d]	928-96-1	17.29	67	156.5	1.61	0 - 0.888
3-Octanol [b] (IS)	20296-29-1	17.54	59	-	-	-
Ethyl octanoate [b]	106-32-1	19.12	127	208.5	3.81	0 - 2.124
Methyl nonanoate [b] (IS)	1731-84-6	20.33	74	-	-	-
Linalool [b]	78-70-6	22.40	93	198	2.97	0 - 0.498
5-Methylfurfural [b]	620-02-0	23.03	109	187	0.67	0 - 1.475 [j]
Terpinen-4-ol [b]	2438-10-0	23.80	93	209	3.26	0 - 0.665
Ethyl decanoate [c]	110-38-3	25.63	101	241.5	4.79	0 - 0.931
α-Terpineol [b]	10482-56-1	26.68	59	217.5	2.98	0 - 0.433
β-Citronellol [b]	106-22-9	28.69	69	224	3.91	0 - 1.563 [j]
Nerol [b]	106-25-2	29.55	69	225	3.56	0 - 7.838 [j]
β-Damascenone [e]	23726-93-4	29.98	69	274-275	4.21	0 - 0.425 [j]
β-Phenylethyl acetate [b]	103-45-7	29.85	104	232.6	2.30	0 - 1.531
α-Ionone [b]	127-41-3	30.73	93	259-263	3.85	0 - 0.228 [j]
Benzyl alcohol [a]	100-51-6	31.47	79	205.3	1.10	0 - 1.563 [j]
trans-Whiskey lactone [a]	80041-01-6	31.68	99	260.63	2.00	0 - 0.868 [j]
β-Phenylethyl alcohol [c]	60-12-8	32.32	91	218.2	1.36	0 - 7.838 [j]
β-Ionone [b]	79-77-6	33.00	177	262.93	3.84	0 - 0.240
cis-Whiskey lactone [a]	80041-00-5	33.38	99	260.63	2.00	0 - 0.682 [j]
γ-Nonalactone [a]	104-61-0	35.10	85 [h]	243	2.08	0 - 0.413
4-Ethylguaiacol [f]	2785-89-9	35.27	137 [h]	236.5	2.38	0 - 0.868 [j]
Octanoic acid [g]	124-07-2	36.22	60	239	3.05	0 - 4.656 [j]
Ethyl cinnamate [c]	103-36-6	37.60	131	271	2.99	0 - 0.825
Eugenol [a]	97-53-0	38.47	164 [h]	253.2	2.27	0 - 0.400
4-Ethylphenol [a]	123-07-9	38.76	107 [h]	217.9	2.58	0 - 0.803
3,4-dimethylphenol [g] (IS)	95-65-8	39.78	107 [h]	-	-	-
4-Vinylphenol [f]	2628-17-3	43.53	120 [h]	209.22	2.41	0 - 0.432
Vanillin [a]	148-53-8	46.87	151 [h]	285	1.21	0 - 0.903
Methyl vanillate [f]	3943-74-6	47.65	151 [h]	286	1.82	0 - 0.198
Ethyl vanillate [f]	617-05-0	48.19	196 [h]	292	2.31	0 - 0.733

IS, internal standard.
Manufacturers: [a] Aldrich; [b] Fluka; [c] Merck; [d] Sigma; [e] Firmenich; [f] Lancaster; [g] Scharlau.
[h] Determined in SIM mode.
[i] Hydrophobic constants obtained from EPI Suite (EPA).
[j] In some wines the linear range did not include the whole range of concentration assayed.

HS-SPME procedure

A 40 µL aliquot of internal standard solution (400 mg L^{-1} 3,4 dimethylphenol, 10 mg L^{-1} 3-octanol and 2.5 mg L^{-1} methyl nonanoate) and 2.3 g of NaCl were added to each vial of reconstituted wine and the vials were sealed with PTFE septa (Supelco). The most appropriate of the internal standards was chosen by taking into consideration their stability during the experiments (low variations in absolute area due to wine matrix, time and volatile concentration added). For 3,4-dimethylphenol within the same wine the relative standard deviation (RSD) was lower than 8% for wines 1,2,4 and 5 and 15% for wine 3. However, for 3-octanol and methyl nonanoate the variation percentages were higher. In addition, the variations between wines were also lower for 3,4-dimethylphenol (< 20%) than for the other two internal standards (> 30%). Therefore 3,4-dimethylphenol was chosen in order to avoid as much as possible the correction of matrix effects, which was the main objective of this study.

The extraction procedure was performed automatically using a CombiPal system (CTC Analytics AG, Zwingen, Switzerland) with a 50/30 µm DVB/CAR/PDMS fibre of 2 cm length (Supelco). Samples were pre-incubated for 10 min at 50 ºC and extraction was performed in the headspace of each vial for 30 min at 50 ºC. Desorption was performed in the injector of the GC system (Agilent 6890N) in splitless mode for 1.5 min at 270 ºC. After each injection the fibre was cleaned for 30 min to avoid any memory effect. All analyses were performed in duplicate (one injection per sample vial).

GC/MS analysis

Agilent MSD ChemStation software was used to control the system. For separation a Carbowax 20M fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness; Quadrex, Woodbridge, CT, USA) was used with helium as carrier gas at a flow rate of 1 ml *x* min^{-1}. The oven temperature was initially held at 40 ºC for 5 min, then increased at 4 ºC min^{-1} to 240 ºC and held for 15 min.

In the MS system (Agilent 5973N) the temperatures of the manifold and transfer line were 150 and 230 ºC respectively. Electron impact mass spectra were recorded at an ionization voltage of 70 eV and an ionization current of 10 µA. Acquisitions were performed in scan mode (from 35 to 450 amu) and in selected ion mode (SIM) for some

specific compounds. The signal corresponding to a specific ion of quantification was calculated by the data system. **Table 1** gives the retention time, ion of quantification and detection mode, boiling point, hydrophobic constant and linear concentration range assayed for each volatile compound studied. Quantitative data were obtained by calculating the relative peak area in relation to that of the internal standard (3,4-dimethylphenol).

Chemical Matrix Composition

Total nitrogen, free amino acids and peptides

Total nitrogen was determined by the Kjeldahl method using a heating digestor unit, a SMS Scrubber and an UDK-142 automatic distillation unit (Velp Scientifica, Usmate, Italy).

Free amino acids and peptides plus free amino acids were determined by methods 5 and 1, respectively, published by Doi et al., 1981 A DU 70 spectrophotometer (Beckman Coulter, Brea, CA, USA) was used for both determinations.

Neutral polysaccharides and residual sugars

Neutral polysaccharides were determined by the phenol/sulfuric acid method according to Segarra et al., 1995 Residual sugars (glucose and fructose) were determined following the OIV method (1990).

Total polyphenols

Total polyphenols were determined by the Folin-Ciocalteau method and spectrophotometric measured at 670 nm (Singleton et al., 1965).

Total acidity and pH

Total acidity was determined by titration with 0.05 mol L^{-1} NaOH. pH was determined using a pHmeter (Mettler Toledo, Barcelona, Spain).

Statistical analysis

Linear regressions, to establish the calibration curves of each aroma compound in the five types of reconstituted wine and the control wine, and a lack-of-fit test, to judge the adequacy of the models, were performed. In addition, for each aroma compound the slope from the calibration curve of each wine was compared with that of the control wine. Statgraphics Centurion XV Version 15.2 (Manugistics, Rockville, MD, USA) was used for data processing.

Results and discussion

Non-volatile wine matrix composition

The results obtained from the analysis of wine matrix components (amino acids, peptides, total nitrogen, residual sugars, total polyphenols and neutral polysaccharides) and some physicochemical characteristics (total acidity, pH and weight of non-volatile residue) of the five wines under study are presented in **Table 2**. The % (w/w) of wine residue (compared with the whole volume of wine in the vial) after lyophilisation was calculated as the average residue weighed in 12 vials of the same type of wine. The low deviation of this parameter (RSD < 3.25 %) showed that the lyophilisation process was very reproducible for all wines. As can be seen, the non-volatile residue was lowest for the white (1.9 % w/w) and sparkling (1.8 % w/w) wines. The sweet wine showed the highest non-volatile residue (34.6 % w/w), mainly because of its high content of sugars. In addition, this wine showed the highest levels of nitrogen compounds (total nitrogen, amino acids and peptides). However, the sweet wine presented lower total acidity (3.07 g tartaric acid L^{-1}) and consequently higher pH (4.59) compared with the other wines. Besides the sweet wine, there was also a markedly higher level of residual sugars (9.34 g L^{-1}) in the aged-red wine compared with the other non-sweet wines. In addition, the older wines (aged-red wine and mainly sparkling wine) showed the highest peptide contents. The release of peptides due to the slow hydrolysis of proteins during wine aging has been extensively described (Martínez-Rodríguez et al., 2002; Martínez-Rodríguez & Polo, 2000). The white and sparkling wines showed the lowest polyphenol contents (230 and 125 mg L^{-1} gallic acid, respectively), while, as expected, the young- and aged-red wines showed the highest values (1820 and 2142 mg L^{-1} gallic acid, respectively). Besides the sweet wine, which showed, as stated above, the highest pH (4.59), the pH of the other wines was fairly similar, ranging between 3.02 for the

Table 2. Chemical composition of wine matrices

Component	White	Young-red	Aged-red	Sparkling	Sweet
Non-volatile residue (g)	0.145 (0.005)	0.170 (0.004)	0.213 (0.006)	0.136 (0.004)	3.177 (0.039)
% Non-volatile residue (% w/w)	1.9	2.2	2.7	1.8	34.6
pH	3.2 (0.01)	3.48 (0.03)	3.55 (0.04)	3.02 (0.01)	4.59 (0.01)
Total acidity (mg tartaric acid L^{-1})	5.82 (0.03)	5.71 (0.03)	5.28 (0.00)	5.54 (0.05)	3.07 (0.03)
Total polyphenols (mg gallic acid L^{-1})	230 (4)	1820 (21)	2142 (220)	125 (6)	1088 (31)
Neutral polysaccharides (mg mannose L^{-1})	1816 (31)	3019 (161)	5754 (80)	2795 (114)	360583 (4256)
Residual sugars (mg L^{-1})	3502 (96)	4633 (74)	9337 (29)	4913 (124)	708285 (17325)
Total nitrogen (mg L^{-1})	195.6 (2.4)	104.6 (7.5)	255.4 (1.4)	174.2 (0.6)	929.1 (29.4)
Amino acids + peptides (mg N L^{-1})	52.9 (0.8)	43.3 (0.1)	74.1 (6.7)	62.1 (2.7)	240.6 (12.9)
Amino acids (mg N L^{-1})	27.6 (1.5)	13.6 (0.1)	33.3 (2.0)	23.3 (0.6)	97 (1.6)
Peptides (mg N L^{-1})	25.4	29.7	40.8	38.7	143.6

Values are average of two determinations, except for non-volatile residue (average of 12 lyophilised vials), with SD in parentheses.

sparkling wine and 3.55 for the aged-red wine. These results show great differences in composition among the five types of wine, which may distinctively affect the volatility of the aroma compounds.

Comparison between the regression parameters calculated in the reconstituted and control wines

The influence of ethanol on the volatility of aroma compounds was not considered in this study, since it has been extensively demonstrated (Escalona et al., 1999, Hartmann et al., 2002; Robinson et al., 2009; Rodríguez-Bencomo et al., 2002; Conner et al., 1994). Therefore the ethanol concentration was kept the same in all reconstituted and control wines.

To evaluate the effect of the whole non-volatile composition on the volatility of the aroma compounds, regression lines for the 36 volatile compounds using two replicates at five levels of concentration for each of the five reconstituted wines and the control wine were calculated. In total, 216 regression lines with five points and in duplicate were determined for this study. The slopes of the regression lines obtained for the five reconstituted wines were compared with the slopes calculated for the same compounds in the control wine comprising ethanol and tartaric acid, considering that the latter did not show any matrix effect.

The slopes of the regression lines obtained with the control and reconstituted wines are shown in **Table 3**. The table also shows the residual standard deviation (*s*) and the determination coefficient (R^2) which are estimators of the adequacy of the regression models. In addition, to judge the adequacy of the linear models, the *F* ratio for lack of fit was calculated (Massart et al., 1990). As can be seen, in general, most of the studied aroma compounds showed R^2 higher than 0.99 and very low values of *s*; in fact, *s* expressed as a percentage of the mean value (*s/y*) was lower than 15 % for most compounds (data not shown).

The comparison between the slopes for the aroma compounds in the reconstituted and control wines is also shown in **Table 3**. The slopes indicated in bold were statistically significantly different between the two types of wine after application of the two-sample

t test. In general, in the reconstituted aged-red wine, a higher number of volatile compounds showed differences in their slopes compared with the control wine, while the white wine showed the lowest differences in slopes. Besides the type of wine matrix composition, depending on the type of aroma compound, greater or lesser differences compared with the control wine were also noted. For example, some chemical groups such as C13 norisoprenoids and some volatile phenols, lactones and furanic compounds exhibited important differences in the slopes in most of the reconstituted wines compared with the control wine. Most of them have been described as key aroma compounds in different types of wines (Chatonnet et al., 1992, Ferreira et al., 2004; Mendes-Pinto 2009; Pollnitz et al., 2000). In addition, the slopes of other compounds such as the esters ethyl decanoate and isoamyl acetate, benzyl alcohol, terpinen-4-ol, and the benzenic compound methyl vanillate showed significant differences in the reconstituted wines compared with the control wine. However, some chemical groups such as esters and alcohols did not present such large differences between reconstituted and control wines. These results show an interaction between the wine non-volatile composition and the aroma compounds that depends not only on the wine matrix composition but also on the type and physicochemical characteristics of the aroma compounds.

Interaction between non-volatile composition and aroma compounds

To better understand the interaction between the aroma compounds and the non-volatile composition, **Table 4** shows the results of the comparison of the slopes of the reconstituted and control wines expressed as a percentage. This value can be negative or positive depending on whether the slope was lower or higher respectively than that calculated in the control wine. In this table, slope variations higher than 10% that showed statistically significant differences compared with the control wine are indicated in bold.

Table 3. Slope, determination coefficient (R^2) and residual standard deviations (s) of calibrations in reconstituted wines and control wine

Compound	White			Young-red			Aged-red			Sparkling			Sweet			Control		
	Slope	R^2	*s*	*Slope*	R^2	*s*	*Slope*	R^2	*s*	*Slope*	R^2	*s*	*Slope*	R^2	*s*	*Slope*	R^2	*s*
Esters																		
Ethyl butyrate	0.678	0.984	0.089	0.776	0.996	0.045	**0.913**	0.995	0.061	0.693	0.989	0.073	0.796	0.985	0.099	0.729	0.994	0.041
Ethyl 2-methylbutyrate	1.364	0.980	0.109	**1.677**	0.994	0.066	**2.032**	0.992	0.093	1.470	0.987	0.094	**1.798**	0.982	0.137	1.445	0.983	0.082
Ethyl hexanoate	**3.931**	0.983	0.879	4.230	0.971	1.200	4.300	0.972	0.079	**3.995**	0.986	0.770	4.772	0.981	0.099	4.476	0.999	0.167
Ethyl octanoate	**6.518**	0.980	1.456	7.085	0.993	0.734	**8.326**	0.989	1.362	**6.535**	0.991	0.823	7.665	0.989	1.199	7.359	0.995	0.595
Ethyl decanoate	**10.971**	0.974	1.161	17.377	0.987	1.500	**14.816**	0.989	0.922	**16.573**	0.995	0.622	**15.434**	0.998	0.433	21.275	0.990	0.628
Ethyl cinnamate	**3.316**	0.996	0.126	3.911	0.995	0.154	**3.374**	0.991	0.185	4.148	0.991	0.231	4.245	0.992	0.223	4.090	0.985	0.290
Isobutyl acetate	0.278	0.985	0.016	**0.337**	0.996	0.010	**0.383**	0.993	0.014	0.303	0.994	0.010	0.347	0.980	0.023	0.310	0.993	0.009
Butyl acetate	1.090	0.984	0.069	**1.199**	0.996	0.030	**1.442**	0.993	0.054	1.074	0.993	0.045	**1.269**	0.986	0.074	1.127	0.995	0.029
Isoamyl acetate	**1.122**	0.965	0.242	**1.210**	0.996	0.074	**0.670**	0.990	0.101	**1.063**	0.988	0.132	1.337	0.983	0.198	1.317	0.993	0.095
Hexyl acetate	2.827	0.959	0.971	**3.130**	0.990	0.496	**3.574**	0.988	0.670	2.686	0.985	0.553	**3.301**	0.980	0.795	2.888	0.998	0.146
β-Phenylethyl acetate	**6.323**	0.993	0.564	6.152	0.997	0.331	6.103	0.999	0.213	**5.386**	0.996	0.359	**6.255**	0.996	0.359	5.906	0.996	0.269
Alcohols																		
1-Hexanol	0.304	0.984	0.060	**0.335**	0.996	0.028	**0.358**	0.993	0.047	**0.286**	0.990	0.043	0.320	0.986	0.058	0.314	0.996	0.022
trans-3-Hexen-1-ol	**0.098**	0.988	0.006	0.107	0.998	0.003	0.117	0.996	0.004	**0.093**	0.993	0.004	0.105	0.986	0.008	0.110	0.983	0.007
cis-3-Hexen-1-ol	0.103	0.985	0.008	0.117	0.997	0.003	0.126	0.997	0.004	**0.100**	0.993	0.005	0.112	0.990	0.007	0.119	0.985	0.006
Benzyl alcohol	**0.117**	0.995	0.006	**0.087**	0.992	0.008	**0.104**	0.996	0.006	**0.077**	0.986	0.008	0.097	0.996	0.006	0.089	0.987	0.012
β-Phenylethyl alcohol	0.269	0.991	0.097	0.224	0.994	0.089	0.250	0.984	0.167	**0.200**	0.992	0.084	0.232	0.995	0.077	0.257	0.997	0.025
Terpenes																		
Linalool	2.228	0.971	0.132	**1.851**	0.996	0.034	2.124	0.992	0.069	**1.260**	0.987	0.049	2.015	0.989	0.072	2.139	0.991	0.065
Terpinen-4-ol	1.740	0.971	0.137	**1.392**	0.991	0.059	**1.465**	0.991	0.069	**1.130**	0.991	0.051	**1.262**	0.985	0.071	1.717	0.987	0.076
α-Terpineol	2.618	0.984	0.104	**2.449**	0.983	0.091	2.594	0.993	0.069	2.515	0.985	0.093	**1.706**	0.994	0.041	2.733	0.988	0.084
β-Citronellol	**2.862**	0.991	0.070	**1.733**	0.994	0.045	**1.939**	0.994	0.053	1.280	0.991	0.048	2.236	0.992	0.078	2.191	0.994	0.059
Nerol	1.593	0.982	0.090	**0.936**	0.996	0.023	**1.244**	0.983	0.064	0.501	0.988	0.023	1.718	0.996	0.420	1.638	0.993	0.045

Table 3. *Continued.*

Compound	White			Young-red			Aged-red			Sparkling			Sweet			Control		
	Slope	R^2	*s*	*Slope*	R^2	*s*	*Slope*	R^2	*s*	*Slope*	R^2	*s*	*Slope*	R^2	*s*	*Slope*	R^2	*s*
C13 norisoprenoids																		
β-Damascenone	10.153	0.993	0.169	**7.999**	0.996	0.013	**7.883**	0.993	0.174	**7.671**	0.996	0.141	**8.810**	0.999	0.066	9.858	0.993	0.189
α-Ionone	8.599	0.991	0.109	**7.573**	0.993	0.101	**7.039**	0.998	0.044	**7.105**	0.996	0.068	**7.456**	0.997	0.057	8.340	0.996	0.067
β-Ionone	**19.092**	0.995	0.235	**19.058**	0.999	0.088	**16.697**	0.999	0.081	17.602	0.999	0.094	**18.490**	0.998	0.131	17.408	0.998	0.100
Volatile phenols																		
4-Ethylguaiacol	**1.958**	0.999	0.023	**1.780**	0.999	0.021	**1.751**	0.999	0.013	**1.647**	0.999	0.021	**1.751**	0.999	0.031	1.879	0.999	0.023
Eugenol	**0.591**	0.997	0.008	**0.613**	0.997	0.010	**0.553**	0.993	0.013	**0.599**	0.996	0.011	**0.594**	0.995	0.012	0.750	0.996	0.010
4-Ethylphenol	1.168	0.997	0.030	**1.153**	0.999	0.005	1.176	0.999	0.009	**1.123**	0.999	0.009	**1.146**	0.999	0.017	1.179	0.999	0.013
4-Vinylphenol	**0.087**	0.998	0.001	**0.019**	0.994	0.000	**0.030**	0.981	0.001	**0.090**	0.996	0.001	**0.067**	0.989	0.002	0.113	0.997	0.001
Benzenic compounds																		
Vanillin	0.004	0.986	0.000	0.006	0.994	0.000	0.005	0.994	0.000	**0.007**	0.985	0.000	0.005	0.993	0.000	0.005	0.930	0.001
Methyl vanillate	**0.014**	0.988	0.000	**0.019**	0.991	0.000	**0.016**	0.984	0.000	0.023	0.976	0.000	**0.015**	0.991	0.000	0.021	0.938	0.001
Ethyl vanillate	**0.010**	0.991	0.000	0.013	0.990	0.000	**0.011**	0.986	0.001	0.018	0.967	0.002	**0.011**	0.987	0.001	0.015	0.971	0.001
Lactones/ furanic compounds																		
5-Methylfurfural	**0.569**	0.988	0.053	**0.540**	0.997	0.018	**0.553**	0.992	0.050	**0.484**	0.996	0.028	**0.513**	0.986	0.063	0.427	0.996	0.021
trans-Whiskey lactone	**0.901**	0.997	0.021	**0.714**	0.998	0.019	**0.656**	0.996	0.022	**0.621**	0.998	0.016	**0.826**	0.998	0.016	0.786	0.999	0.013
cis-Whiskey lactone	**0.847**	0.997	0.013	**0.699**	0.999	0.011	**0.663**	0.998	0.012	**0.632**	0.999	0.009	**0.821**	0.998	0.012	0.778	0.999	0.010
γ-Nonalactone	**0.903**	0.997	0.012	**0.886**	0.999	0.008	**0.824**	0.999	0.006	**0.852**	0.999	0.003	**1.088**	0.998	0.011	1.025	0.999	0.005
Acids																		
Octanoic acid	**0.753**	0.996	0.101	0.578	0.998	0.067	0.609	0.996	0.115	0.541	0.998	0.064	**0.760**	0.998	0.085	0.516	0.931	0.181

Slopes of the reconstituted wines that are statistically significantly different ($P < 0.05$) from those of the control wine are indicated in bold.

As can be seen in **Table 4**, the main effect observed is a reduction in the slopes calculated in the reconstituted wines compared to the control wine. This reduction could be considered as a retention effect of certain volatile by the non-volatile wine matrix composition, as has been observed previously in model systems (Dufour & Bayonove, 1999; Hartmann et al., 2002; Dufour & Bayonove, 1999b; Dufour & Sauvaitre, 2000; Escalona et al., 2001). Interestingly, this effect was higher in the case of the reconstituted sparkling wine, which for some esters such as ethyl hexanoate and ethyl octanoate and the terpenic compound nerol showed between 11 % and 69 % lower slopes than the control wine. Although none of the non-volatile compounds determined in the wines was in higher proportion in this type of wine compared with the other four wines (**Table 2**), the reconstituted sparkling wine showed quite a large amount of nitrogen compounds such as amino acids, peptides and total nitrogen. The latter parameter could also indirectly indicate a relevant amount of protein, specifically mannoproteins from yeast origin, very abundant in aged sparkling wines (Nuñez et al., 2005), which have been found to specifically bind several types of aroma compound (Chalier et al., 2007). In addition, the aged-red wine showed lower slopes for many volatile compounds compared with the control wine. These differences in slopes ranged between 12 % and 73 % lower than the control for *β*-citronellol and vinylphenol respectively. The younger wines, i.e. the white and the young-red wine, showed a smaller retention effect. Surprisingly, in spite of the higher complexity of the sweet wine composition, it did not show the expected higher retention effect. It is also important to underline that the reduction in the slopes (or retention effect) noted for many volatile compounds in the reconstituted wines compared with the control wine was much higher than the reduction shown in some recent studies performed in model wine systems supplemented with glucose, catechin, glycine or proline or a combination thereof (Robinson et al., 2009). This indicates large differences and possibly an undervaluation of the retention effect observed when studying wines supplemented with a reduced number of matrix components compared with considering the whole and truly non-volatile composition of the wines.

Table 4. Percentage of slope variation of the volatile compounds in each wine matrix compared with slopes obtained in control wine

Compound	White	Young-red	Aged-red	Sparkling	Sweet
Esters					
Ethyl butyrate	-7	6	**25**	-5	9
Ethyl 2-methylbutyrate	-6	**16**	**41**	2	**24**
Ethyl hexanoate	**-12**	-5	-4	**-11**	7
Ethyl octanoate	**-11**	-4	**13**	**-11**	4
Ethyl decanoate	**-48**	-18	**-30**	**-22**	**-27**
Ethyl cinnamate	**-19**	-4	**-18**	1	4
Isobutyl acetate	**-10**	9	**24**	-2	12
Butyl acetate	-3	6	**28**	-5	**13**
Isoamyl acetate	**-15**	-8	**-49**	**-19**	2
Hexyl acetate	-2	8	**24**	-7	**14**
β-Phenylethyl acetate	7	4	3	-9	6
Alcohols					
1-Hexanol	-3	7	**14**	-9	2
trans-3-Hexen-1-ol	**-11**	-3	6	**-15**	-5
cis-3-Hexen-1-ol	-13	-2	6	**-16**	-6
Benzyl alcohol	**31**	-2	**17**	**-13**	9
β-Phenylethyl alcohol	5	-13	-3	**-22**	-10
Terpenes					
Linalool	4	**-13**	-1	**-41**	-6
Terpinen-4-ol	1	**-19**	**-15**	**-34**	**-26**
α-Terpineol	-4	**-10**	-5	-8	**-38**
β-Citronellol	**31**	**-21**	**-12**	**-42**	2
Nerol	-3	**-43**	**-24**	**-69**	5
C13 norisoprenoids					
β-Damascenone	3	**-19**	**-20**	**-22**	**-11**
α-Ionone	3	-9	**-16**	**-15**	**-11**
β-Ionone	10	9	-4	1	6
Volatile phenols					
4-Ethylguaiacol	4	-5	-7	**-12**	-7
Eugenol	**-21**	**-18**	**-26**	**-20**	**-21**
4-Ethylphenol	-1	-2	0	-5	-3
4-Vinylphenol	**-23**	**-83**	**-73**	**-20**	**-41**
Benzenic compounds					
Vanillin	-20	20	0	**40**	0
Methyl vanillate	**-33**	-10	**-24**	10	**-29**
Ethyl vanillate	**-33**	**-13**	**-27**	20	**-27**
Lactones/furanic compounds					
5-Methylfurfural	**33**	**26**	**30**	**13**	**20**
trans-Whiskey lactone	**15**	-9	**-17**	**-21**	5
cis-Whiskey lactone	9	**-10**	**-15**	**-19**	6
γ-Nonalactone	**-12**	**-14**	**-20**	**-17**	6
Acids					
Octanoic acid	**46**	12	18	5	**47**

Values higher than 10 % that are statistically significantly different ($P < 0.05$) between the wine matrix and the control wine are indicated in bold

In addition to the retention effect, an increase in the slope in the reconstituted wines compared with the control wine was also noticed for some volatiles. This effect means an increase in the volatility of some compounds in the presence of specific non-volatile compounds, which is also called a "salting-out" effect. It can be seen in **Table 4** that the compositionally more complex reconstituted aged-red and sweet wines seemed to induce this effect to a higher extent. It is interesting to underline that this effect seems to be more evident for certain esters, such as ethyl 2-methylbutyrate, butyl acetate, and hexyl acetate, and other compounds such as 5-methylfurfural, all of which have a very low boiling point or a low log *P* value (**Table 1**). Mono- and disaccharides in solution are known to absorb water molecules, thus decreasing the amount of free water in the matrix and increasing the concentration of aroma compounds in the remaining available free water, which in turns affects the apparent partition equilibrium of the volatile compounds in favour of the gas phase (Delarue & Giampaoli, 2006). In addition to mono- and disaccharides, other small soluble compounds such as amino acids may also induce a salting-out effect in wine (Pozo-Bayón et al., 2009).

Depending on the aroma chemical class and examining the differences observed between the slopes in the reconstituted and control wines (**Table 4**), it was possible to observe some similar trends between compounds from the same chemical class and their behaviour in the five reconstituted wines.

Esters: In general, in the white and sparkling wines a reduction in the slope for many esters compared with the control wine was found. However, the aged-red and sweet wines showed both effects, retention and salting-out, depending on the compound. The higher amount of sugars and other soluble compounds in these wines might be responsible for the observed effect (Delarue et al., 2006).

Among the linear ethyl esters, the most hydrophobic compound, ethyl decanoate, (log *P* = 4.79) showed the highest retention effect in all wines, possibly owing to a higher interaction with the wine matrix. The low polarity of ethyl hexanoate (log *P* = 2.83) and ethyl octanoate (log *P* = 3.81) also seemed to be involved in their higher retention by the wine matrix.

Although, ethyl cinnamate showed a hydrophobic constant (log *P* = 2.99) similar to that of ethyl hexanoate (log *P* = 2.83), the behaviour of the two compounds presented some differences. The π-π interactions of the aromatic cycle with other electron unsaturated systems of the matrix may explain the higher retention of ethyl cinnamate, in the white and aged-red wines (Jung & Ebeler, 2003).

Interestingly, small esters with low boiling points and relatively low log *P* values, such as ethyl butyrate, ethyl 2-methylbutyrate, isobutyl acetate and butyl acetate, generally showed a very low interaction with the studied wine matrices, except for the aged-red wine.

Alcohols: This group of compounds was not affected as much as other chemical groups by the non-volatile composition. The C6 alcohols, 1-hexanol, *cis*-3-hexen-1-ol and *trans*-3-hexen-1-ol showed similar hydrophobic constants (log *P* = 1.61-2.03) and therefore similar behaviour. Only a slight retention effect (15-16 %) for both alkenols in sparkling wine and a salting-out effect (14 %) for 1-hexanol in the aged-red wine were observed. The aromatic alcohols, *β*-phenylethyl alcohol and benzyl alcohol only showed a retention effect in the sparkling wine, particularly the more hydrophobic *β*-phenylethyl alcohol (log *P* = 1.36). However, benzyl alcohol (log *P* = 1.10) presented a salting-out effect in the white (31 %) and aged-red (17 %) wines.

Terpenes: In all reconstituted wines except the white wine, most terpenes showed a retention effect. The slopes calculated in the reconstituted wines were between 13 % and 69 % lower than those in the control wine. The white wine, however, did not show any retention effect, which is in agreement with its simpler matrix composition, more similar to that of the control wine. In the red and sparkling wines, the cyclic terpenes terpinen-4-ol but mainly *α*-terpineol showed a slightly lower retention effect compared with the non-cyclic terpenes linalool, nerol and *β*-citronellol, revealing the important effect of the molecular chemical structure in the interaction with some non-volatile compounds (Heng et al., 2004; Semenova et al., 2002). However, in the sweet wine the non-cyclic terpenes linalool, nerol and *β*-citronellol did not show any effect, probably owing to the retention effect compensating the salting-out effect of sugar (Robinson et al., 2009).

Interestingly, the aged red-wine showed lower retention than the young-red wine. This may be due to differences in the type of polyphenols, which have been shown to interact with terpenic compound in ethanolic or aqueous solutions. Polymeric polyphenols, more abundant in older wines, have lower retention capacity than monomeric polyphenols, as reported by Dufour et al.,1999a who observed higher retention of limonene by catechin than by tannin.

Although the main effect observed for terpenes was retention by the non-volatile composition, *β*-citronellol in the reconstituted white wine showed a higher slope than in the control wine, i.e. an increase in its volatility or a salting-out effect was observed. No explanation based on the compositional parameters analysed in this wine seems to account for this effect; however, other non-analysed matrix chemical components may be the responsible for the observed effect.

C13 norisoprenoids: Among the C13 norisoprenoids studied, the most hydrophobic, *β*-damascenone (log *P* = 4.21) showed the highest retention effect in all reconstituted wines except the white wine. The retention effect was lower for *α*-ionone (log *P* = 3.85). However, *β*-ionone, with almost the same log *P* and boiling point as *α*-ionone, did not show any significant retention effect. This shows the high specificity of some interactions between these compounds and some non-volatile compounds of the wine matrix.

Volatile phenols: Volatile phenols presented similar hydrophobic constants, which ranged from log *P* = 2.27 for eugenol to log *P* = 2.58 for 4-ethylphenol. Among them, 4-ethylphenol and 4-ethylguaiacol did not show any important effect due to the matrices studied. However, eugenol and 4-vinylphenol presented a noticeable retention effect in all wines. For eugenol, this effect was similar in all wines (between 18 and 26 %). However, 4-vinylphenol showed great differences among the wine matrices. While the white and sparkling wines showed a slight retention effect (~20 %), the red wines showed a strong retention effect (slope 73-83 % lower than in the model solution). This strong retention effect for the red wines could be due to important π-π interactions resulting from the high content of polyphenols in these wines (Jung & Ebeler, 2003). Vinylphenols have been associated with off-flavours produced by spoilage

microorganisms in red wines (Chatonnet et al., 1992), and on the basis of these results the polyphenol content of wines might contribute to a decrease in their sensory effect. The sweet wine, with a lower content of total polyphenols and a higher content of sugars than red wines, may compensate the high retention effect of polyphenols with the salting-out effect due to the high content of sugars. The lower retention in the white and sparkling wines could be due to the low concentrations of polyphenols found in these wines (230 and 125 mg gallic acid L^{-1} respectively).

Benzenic compounds: Methyl vanillate and ethyl vanillate showed retention effect in most of the studied wines, which could be due to their relatively high hydrophobic constants (log *P* = 1.82 and 2.32 respectively). However, vanillin showed a statistically significant effect only for the sparkling wine (40 %). The hydrophobic constant of vanillin (log *P* = 1.21) is the lowest of the three compounds, which could explain its minor hydrophobic interactions compared with the respective methyl and ethyl esters.

Lactones and furanic compounds: The furanic compound 5-methylfurfural showed a salting-out effect in all wine matrices, exhibiting in all cases higher slope in the reconstituted wine than in the control wine. This compound presented the lowest log *P* value (0.67) of all volatile compounds under study. In addition, it exhibited a salting-out effect independently on the wine type, thus confirming the great importance of the hidrophobicity of the molecule in explaining the retention effect with the non-volatile wine matrix compounds. The behaviour of both whiskey lactones was fairly similar in the red and sparkling wines, showing a slight retention effect (9-21 %). In contrast, *trans*-whiskeylactone (15 %) showed a slight salting-out effect in the white wine.

Acids: Only the behaviour of octanoic acid was studied. This compound exhibited a relatively high hydrophobicity (log *P* = 3.05), but only presented statistically significant effects in the white and sweet wines. In both wines a salting-out effect was observed, showing a 46-47 % increase in its slope compared with the control wine. Although in the case of the sweet wine the higher amount of sugars might be the responsible for the observed effect, in the case of the white wine no explanation based on the composition parameters analysed seems to account for this effect.

Principal component analysis

As evidenced above, the interaction effect (retention or salting-out) observed for the aroma compounds in the different wine matrices depended strongly on the type of matrix and on the physicochemical characteristics of the volatile compound. Therefore, to obtain straightforward relationships between the behaviour of a compound and the composition of each matrix is very difficult. Nonetheless, in order to gain insight into the relationships between the type of aroma compound and the interactions with the wine non-volatile composition, a principal component analysis (PCA) considering the slopes for all volatile compounds in the six wines and their compositional parameters was carried out. From this treatment, four main principal components (PCs) were obtained. The first principal component (PC1) explained 33.27% of the data variation and showed high correlation with hexyl acetate (-0.736), β-phenylethyl acetate (-0.837), linalool (-0.715), nerol (-0.761), methyl vanillate (0.861), ethyl vanillate (0.866) and octanoic acid (-0.743). Moreover, several compositional parameters determined in the matrices were correlated with PC1, such as the non-volatile residue (-0.705), amino acids (-0.727), pH (-0.825) and total nitrogen (-0.728). The second principal component (PC2), explained 27.51 % of data variation and correlated with the volatile compounds, ethyl 2-methylbutyrate (-0.740), isobutyl acetate (-0.765), β-phenylethyl alcohol (0.713), terpinen-4-ol (0.825), β-citronellol (0.791), β-damascenone (0.938), α-ionone (0.981), 4-ethylguaiacol (0.920), *trans*-whiskey lactone (0.808) and *cis*-whiskey lactone (0.749). The third principal component (PC3) explained 22.06 % of data variation and correlated with ethyl cinnamate (0.797) and isoamyl acetate (0.749). Finally, the fourth principal component (PC4) explained a 13.62 % of the data variation and correlated with ethyl decanoate (-0.882), eugenol (-0.822) and 5-methylfurfural (0.801). Therefore only PC1 was correlated with the compositional parameters. **Figure 1** shows the representation of the six types of matrix in the plane defined by PC1 and PC2, which explained 61 % of the data variation. As can be seen, PC1 showed high positive values for the sparkling wine and high negative values for the sweet wine. The control, white and young-red wines exhibited very similar values for PC1, while the aged-red wine was between the above-mentioned wines and the sweet wine. Therefore PC1 mainly shows a separation between wines due to their differences in non-volatile matrix composition. In addition, those volatile compounds positively and negatively correlated

with PC1 showed the highest differences in behaviour depending on the matrix composition.

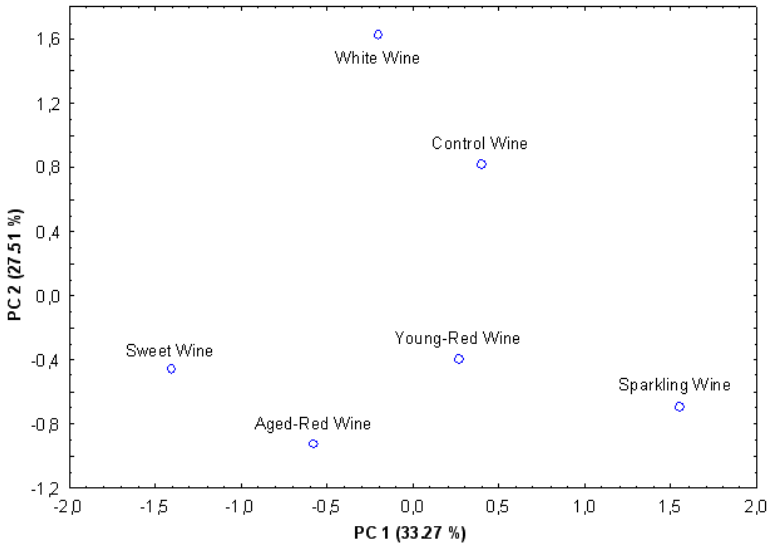


Figure 1. Representation of reconstituted wines in plane defined by first two PCs obtained from PCA considering slopes from all volatile compounds in six wines and their compositional parameters.

PC2, however, showed higher differences between the white and control wines and the other wine types. All volatile compounds associated with PC2 showed a very different behaviour in the white wine than in the other four types of wine. While volatile compounds positively correlated with PC2 showed no effect or a salting-out effect in the white wine, they showed the opposite effect on the other four matrices. In contrast, the compounds ethyl 2-methylbutyrate and isobutyl acetate negatively associated with PC2 showed a slight retention effect in the white wine and the opposite effect in the other four types of wine. Therefore PCA evidenced specific aroma compounds that behaved differently depending on the matrix composition, in which the white wine, compositionally more similar to the control wine, showed the highest differences towards the aroma compounds compared with the other four matrices.

Conclusions

This study has shown that the non-volatile composition of wines strongly influences the volatility of wine aroma compounds. Two opposite effects, a retention effect, i.e. a decrease in the amount of aroma in the headspace, and a salting-out effect, i.e. an increase in the volatility of some aroma compounds, were observed depending on

the non-volatile matrix composition. In addition, the aroma chemical class, in particular its physicochemical properties (volatility and log *P* value), strongly influence this behaviour. On the basis of our results, many odour threshold values calculated in simply hydroalcoholic solutions and usually employed to evaluate the odour importance of specific volatile compounds may have been over- or underestimated. New experiments will be carrying out to verify the importance of these interactions in the sensory aroma perception of wines.

References

Aronson J, and Ebeler SE, Effect of polyphenol compounds on the headspace volatility of flavors. Am J Enol Vitic 55: 13-21 (2004).

Campo E, Ferreira V, Escudero A and Cacho J, Prediction of the wine sensory properties related to grape variety from dynamic-headspace gas chromatography-olfactometry data. J Agric Food Chem 53: 5682-5690 (2005).

Chalier P, Angot B, Delteil D, Doco T and Gunata Z, Interactions between aroma compounds and whole mannoprotein isolated from Saccharomyces cerevisiae strains. Food Chem 100: 22-30 (2007).

Chatonnet P, Dubourdieu D and Boidron JN, Incidence of Fermentation and Aging Conditions of Dry White Wines in Barrels on Their Composition in Substances Yielded by Oak Wood. Sci Aliments 12: 665-685 (1992).

Conner JM, Paterson A and Piggott JR, Interactions between Ethyl-Esters and Aroma Compounds in Model Spirit Solutions. J Agric Food Chem 42: 2231-2234 (1994).

Conner JM, Birkmyre L, Paterson A and Piggott JR, Headspace concentrations of ethyl esters at different alcoholic strengths. J Sci Food Agric 77: 121-126 (1998).

Escalona H, Piggott JR, Conner JM and Paterson A, Effect of ethanol strength on the volatility of higher alcohols and aldehydes. It J Food Sci 11(3): 241-248 (1999).

Escalona H, Homman-Ludiye H, Piggott JR and Paterson A, Effect of potassium bitartrate, (+)-catechin and wood extracts on the volatility of ethyl hexanaote and octanal in ethanol/water solutions. Food Sci Technol-LWT 34(2): 76-80 (2001).

Escudero A, Campo E, Farina L, Cacho J and Ferreira V, Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. J Agric Food Chem 55: 4501-4510 (2007).

Delarue J and Giampaoli P, Carbohydrate-flavour interactions. in Flavour in Food, ed. by Voilley A. and Etiévant P, Woodhead Publishing Limited, Abington, England and CRC Press LLC, Boca Raton, USA, pp. 208-224 (2006).

Doi E, Shibata D and Matoba T, Modified colorimetric ninhydrin methods for peptidase assay. Anal Biochem 118: 173-184 (1981).

Dufour C and Bayonove CL, Interactions between wine polyphenols and aroma substances. An insight at the molecular level. J Agric Food Chem 47: 678-684 (1999).

Dufour C and Bayonove CL, Influence of wine structurally different polysaccharides on the volatility of aroma substances in a model system. J Agric Food Chem 47: 671-677 (1999).

Dufour C and Sauvaitre I, Interactions between anthocyanins and aroma substances in a model system. Effect on the flavor of grape-derived beverages. J Agric Food Chem 48: 1784-1788 (2000).

Ferreira V, López R, Escudero A and Cacho J, Quantitative determination of trace and ultratrace flavour active compounds in red wines through gas chromatographic ion trap mass spectrometric analysis of microextracts. J Chrom A 806: 349-354 (1998).

Ferreira V, Ortín N, Escudero A, López R and Cacho J,. Chemical characterization of the aroma of Grenache rose wines: Aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. J Agric Food Chem 50: 4048-4054 (2002).

Ferreira V, Jarauta I, Ortega L and Cacho J, Simple strategy for the optimization of solid-phase extraction procedures through the use of solid-liquid distribution coefficients Application to the determination of aliphatic lactones in wine. J Chrom A 1025: 147-156 (2004).

Guth H, .Identification of character impact odorants of different white wine varieties. J Agric Food Chem 45: 3022-3026 (1997).

Hartmann PJ, McNair HM and Zoecklein BW, Measurement of 3-alkyl-2-methoxypyrazine by headspace solid-phase microextraction in spiked model wines. Am J Enol Vitic 53: 285-288 (2002).

Heng L, van Koningsveld GA, Gruppen H, van Boekel M, Vincken JP, Roozen JP and Voragen AGJ, Protein-flavour interactions in relation to development of novel protein foods. Trends Food Sci Technol 15: 217-224 (2004).

Jung DM and Ebeler SE, Headspace solid-phase microextraction method for the study of the volatility of selected flavor compounds. J Agric Food Chem 51: 200-205 (2003).

Kotseridis Y and Baumes R, Identification of impact odorants in Bordeaux red grape juice, in the commercial yeast used for its fermentation, and in the produced wine. J Agric Food Chem 48: 400-406 (2000).

Langourieux S and Crouzet JC,. Study of interactions between aroma compounds and glycopeptides by a model system. J Agric Food Chem, 45: 1873-1877 (1997).

Lubbers S, Charpentier C, Feuillat M and Voilley A, Influence of Yeast Walls on the Behavior of Aroma Compounds in a Model Wine. Am J Enol Vitic 45: 29-33 (1994).

Lubbers S, Voilley A, Feuillat M and Charpentier C, Influence of Mannoproteins from Yeast on the Aroma Intensity of a Model Wine. Food Sci Technol-LWT 27(2): 108-114 (1994).

Martínez-Rodríguez AJ and Polo MC, Characterization of the nitrogen compounds released during yeast autolysis in a model wine system. J Agric Food Chem 48: 1081-1085 (2000).

Martínez-Rodríguez, AJ, Carrascosa AV, Martín-Álvarez PJ, Moreno-Arribas V and Polo MC, Influence of the yeast strain on the changes of the amino acids, peptides and proteins during sparkling wine production by the traditional method. J Ind Microbiol Biotechnol 29: 314-322 (2002).

Massart DL, Vandeginste BGM, Deming SN, Michotte Y and Kaufman L. Chemometrics: A Textbook. Elsevier, Amsterdam: (1990).

Mendes-Pinto MM, Carotenoid breakdown products the-norisoprenoids-in wine aroma. Arch Biochem Biophys 483: 236-245 (2009).

Núñez YP, Carrascosa AV, González R, Polo MC and Martínez-Rodríguez AJ, Effect of accelerated autolysis of yeast on the composition and foaming properties of sparkling wines elaborated by a champenoise method. J Agric Food Chem 53: 7232-7237 (2005).

OIV, Recueil des méthodes internationales dánalyse des vins et des mouts. in Complément nº1 à l'edition officielle de juin 1990. Organisation Internationale de la Vigne et du Vin (1990).

Pineau B, Barbe JC, Van Leeuwen C and Dubourdieu D, Which impact for β-damascenone on red wines aroma? J Agric Food Chem 55: 4103-4108 (2007).

Pollnitz AP, Pardon KH and Sefton MA, Quantitative analysis of 4-ethylphenol and 4-ethylguaiacol in red wine. J Chrom A 874: 101-109 (2000).

Pozo-Bayón, M. A., & Reineccius, G. Interactions between wine matrix macro-components and aroma compounds. in Wine Chemistry and Biochemistry, ed. by Moreno-Arribas MV and Polo C. Springer Life Sciences, New York, USA, pp. 417-435 (2009).

Pozo-Bayón MA, Andújar-Ortiz I, Alcaide-Hidalgo JM, Martín-Álvarez PJ and Moreno-Arribas MV, Characterization of commercial inactive dry yeast preparations for enological use based on their ability to release soluble compounds and their behavior toward aroma compounds in model wines. J Agric Food Chem 57: 10784-10792 (2009).

Robinson AL, Ebeler SE, Heymann H, Boss PK, Solomon PS and Trengove RD, Interactions between Wine Volatile Compounds and Grape and Wine Matrix Components Influence Aroma Compound Headspace Partitioning. J Agric Food Chem 57: 10313-10322 (2009).

Rodríguez-Bencomo JJ, Conde JE, Rodríguez-Delgado MA, García-Montelongo F and Pérez-Trujillo JP, Determination of esters in dry and sweet white wines by headspace solid-phase microextraction and gas chromatography. J Chrom A 963: 213-223 (2002).

Segarra I, Lao C, López-Tamames E and De La Torre Boronat MC, Spectrophotometric methods for the analysis of polysaccharide levels in winemaking products. Am J Enol Vitic 46: 564-570 (1995).

Semenova MG, Antipova AS and Belyakova LE, Food protein interactions in sugar solutions. Curr Opin Colloid Interface Sci 7: 438-444 (2002).

Singleton VL and Rossi JA, Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. Am J Enol Vitic 16: 144 – 158 (1965).

Voilley A. Flavour retention and release from the food matrix: an overview. in Flavour in Food, ed. by Voilley A. and Etiévant P, Woodhead Publishing Limited, Abington, England and CRC Press LLC, Boca Raton, USA, pp. 117-132: (2006).

Whiton RS and Zoecklein BW, Optimization of headspace solid-phase microextraction for analysis of wine aroma compounds. Am J Enol Vitic 51: 379-382 (2000).

4. 2 Efecto de la matriz del vino en la liberación del aroma retronasal en condiciones de consumo.

En el estudio previo presentado en el **Apartado 4.1**, se comprobó mediante la técnica de muestreo de espacio de cabeza estático (HS-SPME-GC/MS), que la composición de la matriz vínica afecta la volatilidad de un número representativo de compuestos del aroma del vino. Estas interacciones entre compuestos del aroma y la matriz vínica podrían tener consecuencias tanto en la percepción ortonasal (cuando olemos un vino), como en la percepción retronasal (durante el consumo del mismo).

Sin embargo, las condiciones estáticas empleadas en este estudio, aunque necesarias para evaluar la existencia de interacciones entre compuestos del aroma y compuestos no volátiles de la matriz del vino, no representan completamente la situación dinámica que se produce durante el consumo. Además cuando los alimentos son introducidos en la cavidad bucal, se ven sometidos a un conjunto de cambios debido a las características físico-químicas de la boca (temperatura, pH) y por la acción de distintos parámetros orofisiólogicos (presencia de saliva y microorganismos, flujos de aire, interacción con las mucosas, etc.). Por tanto, para evaluar el efecto de la matriz no volátil del vino en la liberación del aroma y en la composición del aroma retronasal, es necesario realizar estudios *in vivo* que consideren todos estos factores. Pese a que esta investigación está cobrando gran importancia para algunos productos alimentarios, por ej, los derivados lácteos, en el caso del vino este es un área apenas explorada.

Por ello, la primera etapa del trabajo experimental, consistió en el desarrollo de un sistema que permitiera atrapar el aroma liberado durante el consumo. Se trata de un dispositivo de diseño propio que incluye una mascarilla de vidrio que recoge el aire de exhalación durante el consumo y que, conectada a una bomba de vacío, favorece el paso del aroma a través de un polímero adsorbente situado en su interior. El aroma contenido en las trampas poliméricas se desorbió con una mezcla de solventes, se concentró y los extractos de aroma exhalado se analizaron *off-line* mediante el empleo de un GC/MS equipado con un sistema de inyección en frío (CIS, Cool Injection System). El desarrollo de este sistema junto con su aplicación para la evaluación del efecto de la

formulación (etanol, azúcar) de diferentes de sistemas vínicos modelo en la cantidad de aroma liberado durante el consumo forma parte de la **Publicación 2**.

Una vez demostrada la utilidad del sistema para monitorizar cambios en el aroma retronasal debidos a la composición en vinos sintéticos empleando condiciones de consumo, el sistema se empleó para determinar el impacto de la composición de la matriz vínica, empleando para ello cinco vinos de diferente tecnología de elaboración (blanco, espumoso, dulce y tinto joven, tinto crianza), y por tanto distinta composición, en la liberación del aroma retronasal durante el consumo. Para los ensayos *in vivo*, se reclutaron voluntarios ($n = 6$) que fueron previamente entrenados en un procedimiento de consumo definido. Antes de la evaluación, los vinos se ajustaron al mismo contenido de etanol (evitando efecto diferente de este compuesto en la partición de los compuestos del aroma) se aromatizaron con una mezcla de aromas formada por cuatro compuestos volátiles representativos del perfil del aroma del vino (acetato de isoamilo, hexanoato de etilo, linalool, β-feniletanol) (**Publicación 3**).

A pesar de la utilidad del sistema de atrapamiento retronasal (RATD-GC/MS) para monitorizar el aroma liberado durante el consumo del vino, el hecho de que la liberación de aroma pueda variar durante el tiempo de consumo del alimento, requiere de metodologia analítica que permita la monitorizacion la liberación del aroma en tiempo real, que normalmente está mejor relacionado con la evolución en los estímulos sensoriales que se produce durante el consumo. Por ello, en un estudio posterior se decidió emplear una boca artificial que permitía simular en gran medida muchos de los parámetros de la fisiología de la cavidad oral durante el consumo (como la presencia de saliva, flujos de aire o temperatura), acoplada *on line* con un instrumento de PTR-ToF-MS. Esta técnica permite captar la dimensión temporal de la liberación de aroma que se experimenta durante el consumo (monitorización a *tiempo real*) y proporciona una mejor correlación entre la sensación percibida durante el consumo y la concentración y el tipo de molécula responsable (**Publicación 4).** Durante este trabajo se evaluó el efecto de cinco matrices vínicas (espumoso, vino blanco, vino tinto joven, tinto crianza y vino dulce) con muy diferente composición, previamente desaromatizadas y reconstituidas a la misma concentración de etanol (12 % v/v), en la liberación de ocho compuestos del aroma representativos del perfil volátil de un vino en condiciones dinámicas.

A continuación se presentan los resultados de estos trabajos en forma de publicaciones científicas:

Publicación 2: **Carolina Muñoz-González**, Juan J. Rodríguez-Bencomo, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón. "Feasibility and application of a retronasal aroma-trapping device to study in vivo aroma release during the consumption of model wine-derived beverages". *Food Science and Nutrition*, **2014** DOI: 10.1002/fsn3.111

- Además este trabajo fue presentado como comunicación oral titulada "Optimization of a breathing trapping methodology for the off-line GC-MS analysis of retronasal aroma compounds during drinking" en la XII Reunión Científica de la Sociedad Española de Cromatografía y Técnicas Analíticas (SECyTA), Tarragona, España, 14-16 Nov, 2012. Juan J. Rodríguez-Bencomo, **Carolina Muñoz-González**, Amaya Amestoy, M. Victoria Moreno-Arribas, M. Ángeles. Pozo-Bayón.

Publicación 3: **Carolina Muñoz-González**, Pedro J. Martín-Álvarez, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón. "Impact of the nonvolatile wine matrix composition on the in vivo aroma release from wines". *Journal of Agricultural and Food Chemistry*, 62 (1), **2014**, 66-73.

- Además este trabajo fue presentado como comunicación oral titulada "Impact of the non-volatile wine matrix composition on the retronasal aroma release during wine consumption" en la 3th Edition of the International Conference Series of Wine Active Compounds, Beaune, France, 26-28 March, 2014. **Carolina Muñoz-González**, Pedro J. Martín-Álvarez, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón.

Publicación 4: **Carolina Muñoz-González**, Etienne Semon, Gilles Feron, Elisabeth Guichard, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón. "Wine matrix composition affects temporal aroma release as measured by PTR-ToF-MS". Publicación enviada.

Publicación 2. Viabilidad y aplicación de un dispositivo de atrapamiento de aroma retronasal para estudiar la liberación de aroma in vivo durante el consumo de bebidas modelo derivas de vino.

Feasibility and application of a retronasal aroma trapping device to study in vivo aroma release during the consumption of model wine derived beverages

Carolina Muñoz-González, Juan José Rodríguez-Bencomo, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón.

Food Science & Nutrition 2014 DOI: 10.1002/fsn3.111

Abstract:

New types of wine derived beverages are now in the market. However, little is known about the impact of ingredient formulation on aroma release during consumption, which is directly linked to consumer preferences and liking. In this work, the optimization and validation of a retronasal aroma trapping device (RATD) for the *in vivo* monitoring of aroma release was carried out. This device was applied to assess the impact of two main ingredients (sugar and ethanol) in these types of beverages on *in vivo* aroma release. Two aroma trapping materials (Lichrolut and Tenax) were firstly assayed. Tenax provided higher recovery and lower intra- and inter-trap variability. In *in vivo* conditions, RATD provided an adequate linear range ($R^2 > 0.91$) between 0-50 mg L^{-1} of aroma compounds. Differences in the total aroma release were observed in equally trained panelists. It was proven that the addition of sugar (up to 150 mg Kg^{-1}) did not have effect on aroma release, while ethanol (up to 40 mg L^{-1}) enhanced the aroma release during drinking. The RATD is a useful tool to collect real *in vivo* data to extract reliable conclusions about the effect of beverage components on aroma release during consumption. The concentration of ethanol should be taken into consideration for the formulation of wine derived beverages.

Introduction

The increasing interest of consumers in light, fruity and low alcohol beverages have caught the attention of the wine industry, which has found in these demands an interesting source of diversification in new types of wine based products (healthier products with low alcohol content, with added sweeteners, mixed with fruit juices, etc). Therefore, in the coming years, one of the main challenges for the wine sector will be focused on promoting and diversifying their production.

Aroma is one of the most outstanding characteristics determining food preferences and consumption patterns. Understanding the behaviour of aroma molecules in beverages during consumption is necessary for the development of new drinks, which should still taste as good as the reference products. In addition, when producing high quality beverages, it will be important to determine whether the change in one or several ingredients in the formulation of the product, could affect the aroma release pattern and therefore, the sensory characteristics of the product.

Different works have shown the impact of wine matrix composition on aroma release in static conditions (Dufour and Bayonove 1999; Dufour and Bayonove 1999; Dufour and Sauvaitre 2000; Aznar et al. 2004; Pozo Bayón and Reineccius 2009; Robinson et al. 2009; Saenz-Navajas et al. 2010; Munoz-Gonzalez et al. 2011; Rodriguez-Bencomo et al. 2011). Although these types of studies have been very valuable in determining specific interactions between aroma compounds and wine macrocomponents, they were not performed in real consumption situations (drinking conditions). Therefore, aspects of the aroma analysis during the drinking process, which includes the effect of release and transport of the aroma compounds to the olfactory epithelium and other physiological aspects accounted for during drinking (swallowing, breathing, interaction with saliva, adsorption with mucus, etc), which have been shown to have an outstanding effect on aroma release (Buettner et al. 2001; Weel et al. 2003; Weel et al. 2004; Genovese et al. 2009; Deleris et al. 2011; Smyth and Cozzoino 2013) have not been considered in previous studies.

Clark and collaborators (Clark et al. 2011) have recently shown the enhancement effect of ethanol on aroma release when using *in vivo* (API-MS) monitoring of aroma compounds during the consumption of flavoured model beers, which is in disagreement

with the retention effect determined for ethanol in previous studies performed in static conditions (Escalona et al. 1999; Rodriguez-Bencomo et al. 2002; Aznar et al. 2004; Aprea et al. 2007). This fact underlines the necessity for *in vivo* studies to determine the real influence of beverage composition on aroma release during consumption.

To monitor aroma release during drinking, different approaches can be used, mainly based on the on-line monitoring of aroma release by using mass spectrometric techniques (API-MS, PTR-MS) (Lindinger et al. 1998; Taylor and R.S. 2000) or the off-line monitoring by trapping the exhaled breath after swallowing (by the nose or mouth) onto adsorbent polymers (Delahunty et al. 1996; Buettner and Schieberle 2000; Lasekan et al. 2009). The on-line monitoring of aroma release by API-MS and PTR-MS has been proven as a sensitive and very valuable tool allowing the real time monitoring of aroma compounds during eating, permitting the collection of valuable data to compare with the sensory analysis of the same product (Munoz-Gonzalez et al. 2011; Deleris et al. 2013). However, some constraints of this approach are the difficulties in the identification of aroma compounds with the same nominal mass (isobaric compounds), the assignment of fragments of the compound of interest produced during the ionization process, or the identification of aroma compounds when analysing real food samples with complex aroma mixtures (Munoz-Gonzalez et al. 2011; Poinot et al. 2013). Moreover, the sophistication and high price of the required instrumentation for this type of analysis could be considered as an additional drawback. On the other hand, the use of trapping polymers for *in breath* analysis do not provide the temporal profile of aroma release, therefore making the interpretation of the sensory results more difficult. Nonetheless, this technique allows the precise identification of the compound of interest and the possibility of concentrating the breath extract increasing its sensitivity (Buettner and Schieberle 2000).In addition, the relatively low cost of this methodology facilitates its implementation in any laboratory.

Therefore, the objectives of this work, were firstly, to evaluate the feasibility of a retronasal trapping device (RATD) to evaluate aroma release during the consumption of wine derived beverages, and secondly, to apply this methodology to study the influence of two main ingredients (sugar and ethanol) typically used in the formulation of these types of beverages. For the first part of the work, *in vitro* dynamic headspace conditions (purge and trap) were selected to compare the performance of two types of

adsorbents to be used in the RATD, while the validation of the RATD conditions to study aroma release from model wine beverages and its application to evaluate the effect of beverage formulation was performed in real *in vivo* conditions during drinking.

Material and methods

Model wine based beverages

For the *in vitro* dynamic headspace experiments (purge and trap), a model wine made up of ethanol (120 mL L^{-1}), Milli Q water and 3.5 g L^{-1} of tartaric acid was prepared. The pH was adjusted to 3.5 with NaOH (4M). Aromatization was performed with a mixture of six aroma compounds representative of the wine volatile profile (ethyl hexanoate, β-ionone, linalool, guaicol, β-phenylethanol and isoamyl acetate), all of them characterized for having a wide range of physicochemical properties (**Table 1**). The aroma mixture was prepared in absolute ethanol and added to the wine making a final concentration of 1 mg L^{-1}, except for β-phenylethanol and guaicol that were added at concentrations of 15 mg L^{-1} and 4 mg L^{-1} respectively. All the solvents and reactants were purchased from Panreac Química S.A. (Barcelona, Spain).

Table 1. Physicochemical properties of the aroma compounds employed in this study.

Compound	CAS number	MW (g mol^{-1})	BP (ºC)	*log P* (1)
Ethyl hexanoate	123-66-0	144	167	2.83
β-Ionone	8013-90-9	192	262	4.42
Linalool	78-70-6	152	204	3.38
Guaiacol	90-05-1	124	211	1.34
β-Phenylethanol	60-12-8	122	224	1.57
Isoamyl acetate	123-92-2	130	134	2.26

(1) *log P*= log of the water partition coefficient estimated from molecular modeling software EPI Suit (U.S EPA 2000-2007).

For the *in vivo* aroma release experiments, different low alcohol model wine beverages were prepared. To do so, a hydroalcoholic solution composed of ethanol (5 mL L^{-1}), Milli Q water and 3.5 g of tartaric acid were used. The pH was adjusted to 3.5 by using 3.5 g of citric acid. To make a pleasant beverage for the assessors, only an aroma mixture composed of ethyl hexanoate, isoamyl acetate and linalool, all at the same concentration (25 mg L^{-1}) was employed to aromatize the wines. This model wine beverage was coded as MWB-1. In addition, three others types of model wine beverages were produced by varying the content of ethanol and/or sucrose: MWB-2 was prepared

like MWB-1 but adding 15 g L^{-1} of sucrose; MWB-3 was prepared with neither alcohol nor sucrose, and MWB-4 was prepared with sucrose (15 g L^{-1}) but without ethanol. **Table 2** details the composition of the four model wine beverages. All the solvents and reactants employed for these model wines were food-grade and were purchased from Panreac Química S.A.

Table 2. Formulation of the model wine derived beverages.

	Model wine derived beverages			
Composition	MWB-1	MWB-2	MWB-3	MWB-4
Aroma mixture [(1)]	+	+	+	+
Tartaric acid (3.5 g L^{-1})	+	+	+	+
Citric acid (3.5 g L^{-1})	+	+	+	+
Ethanol (5 ml L^{-1})	+	+	-	-
Sucrose (15 g Kg^{-1})	-	+	-	+

[(1)] Aroma mixture constituted by isoamyl acetate, ethyl hexanoate and linalool at the same concentration (25 mg/L). Symbols + and - denote presence or absence of a specific ingredient

Dynamic headspace-GCMS analysis

To select the most appropriate adsorbing material to be used in further experiments with the retronasal aroma trapping device (RATD), dynamic headspace sampling conditions (purge and trap) were selected to better approach the dynamic situation accounted for during the drinking process. Preliminary experiments were performed in order to optimize the extraction conditions. In the end, 100 mL of model wine were placed in a special purge flask (250 mL volume). The sample vessel headspace was flushed with purified nitrogen gas (100 mL min^{-1}) during 4 min at 35 ºC and the purged volatiles were trapped in the selected adsorbent material.

For the trapping material, two different polymers were essayed. The traps were made in the laboratory by using three mL empty plastic cartridges (Agilent Technologies, Santa Clara, USA) filled with 100 mg of Tenax TA 60/80 (Sigma-Aldrich, Steinheim, Germany) or Lichrolut EN (Darmstadt, Germany). The adsorbent material was confined between two polyethylene frits (Supelco, Bellefonte, PA, USA). The volatile compounds trapped on the polymers were extracted with 6 mL (3 mL, twice) of a hexane: diethyl ether (1:1) solution through the Tenax trap or dichloromethane in the case of the Lichrolut. Thirty µL of an internal standard (3-octanol, 25 mg L^{-1}) (Sigma-Aldrich) was added to the extract, which was further

concentrated under N_2 stream to a final volume of 200 µL. Before and after use, the traps were conditioned using 6 mL of the above described organic mixtures and dried under vacuum.

The concentrated extract (2 µL) was injected in splitless mode in the injector port of a Gas Chromatograph Agilent 6890N coupled to a quadrupole Mass Detector Agilent 5973. The injection temperature was set at 270 ºC. Volatile compounds were separated on a Supra-Wax polar capillary column (60 m × 0.25 mm i.d. × 0.50 µm film thickness) from Konik (Barcelona, Spain). Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature was initially held at 50 ºC for 2 min, then increased at 8 ºC min^{-1} to 240 ºC and held for 15 min.

For the MS system (Agilent 5973N), the temperature of the transfer line, quadrupole and ion source were 270, 150 and 230 ºC respectively. Electron impact mass spectra were recorded at 70 eV ionization voltages and the ionization current was 10 µA. The acquisitions were performed in Scan (from 35 to 350 amu) and SIM modes. The identification of compounds was based on the comparison of retention times and mass spectra. The mass spectra were compared with those from NIST 2.0 database. Relative peak areas (RPAs) were obtained by calculating the relative peak area in relation to that of the internal standard. Response factors (RFs) in the MS were calculated by injecting increased concentrations (from 1 to 20 mg L^{-1}) of a mixture of the five aroma compounds (all at the same concentration) using the same chromatographic conditions described above. The calculated RFs were: 12319, 12024, 3849, 10956, 4740 and 27726 for isoamyl acetate, ethyl hexanoate, linalool, guaicol, β-phenyl ethanol and β-ionone respectively.

In vivo aroma trapping using RATD-GCMS analysis

A tailor made retronasal trapping device (RATD) was employed to trap the exhaled breath of the assessors during drinking. This glass device (Pobel, Madrid, Spain) allowed the trapping of exhaled breath during beverage consumption into a polymeric trap thanks to a glass nosepiece coupled to a hollow tube in which the trap was fitted. A vacuum pump connected to a rotameter allowed a steady flow through the trap. A flowmeter allowed us to know the exact flow through the trap. **Figure 1** shows a picture of this device.

Figure 1. Analysis of retronasal aroma release during the consumption of a wine-derived beverage by using the RATD.

Three volunteers (2 male and 1 female) between 26-34 years old previously trained in the retronasal aroma trapping procedure participated in this study. They were instructed not to eat, drink or smoke for 2 hours before the experiments. They had no known illnesses and had self-reported normal olfactory and gustatory functions. Before each experiment, the assessors had to clean their mouths and rinse with a bicarbonate solution. The monitorization of the oral cavity of the panelists for the four compounds of interest was performed before each analysis.

The consumption procedure consisted in two steps. In the first one, 20 mL of the model wine beverage was provided to the panelists using a plastic syringe. The sample was kept in the mouth for 20 seconds while the assessor had their lips closed in order to favor the equilibration of the aroma compounds within the oral cavity (Buettner and Schieberle, 2000). During this time no trapping was performed. After this time, the assessor had to swallow and breathe naturally using the nose through the glass nosepiece for 20 more seconds. During these 20 seconds, volatiles contained in the breath were trapped into the selected trap. The procedure was repeated until they had consumed 100 mL of the model wine based beverages. The same trap was used for a single experiment (corresponding to the trapping of the expiration breath of 100 mL of model wine based beverage). The experiments were carried out in duplicate by using two different traps.

The aroma compounds from the expiration breath trapped onto the trap were desorbed with 6 mL of a hexane/diethyl ether solution (1:1). Thirty µL of an internal standard (3-octanol) were added, and the sample was concentrated using a nitrogen stream to a final volume of 200 µL and analyzed in the GC-MS. A volume of 8 µL of the concentrated breathe extract was injected in a cool injection system unit (CIS)

(Gerstel, Mülheim an der Ruhr, Germany) in the solvent vent mode. These conditions were previously optimized and were: vent time: 0.26 min, vent flow: 80 mL min $^{-1}$, injection speed: 0.5 µL s^{-1}, injection temperature ranged from -80 ºC to 270 °C with a 12 ºC s^{-1} ramp . The variability of the repeatability of the injection mode in these conditions was <5% for the aroma compounds employed in this study. The rest of the analysis was carried out using the same GC-MS conditions described in the section above. Relative peak areas (peak area compound /peak area internal standard) were used to express total aroma release during the *in vivo* analysis.

Statistical analysis

One way ANOVA was used to determine the significant effect of the trapping polymer on the recovery of aroma compounds and to determine the inter-individual effect of the panelists on aroma release performance. Two-way ANOVA was employed to find out the effect of sugar and ethanol on the *in vivo* aroma release during the consumption of the wine based beverages. Least significant difference (LSD) test was used for mean comparison. Linear regression was employed to establish the regression parameters for each aroma compound released after drinking the model wine beverage and the lack of fit test was used to judge the adequacy of the linear models. The STATISTICA program for Windows version 7.1 was used for data processing (StatSoft, Inc., 2005, www.statsoft.com).

Results and discussion

Selection of the most suitable aroma trapping polymer for the in vivo aroma release experiments

Most of the trapping devices described in the literature to monitor *in vivo* or *in vitro* food aroma release are based on the use of Tenax (Buettner and Schieberle 2000; Margomenou et al. 2000; Lasekan et al. 2009) as the adsorbent material to entrap the volatile compounds contained in the so-called exhalation breath through the mouth or through the nose. In the case of wine aroma analysis, other entrapping polymers such as Lichrolut are often used and it has been proven to give very good performance for the isolation of wine volatiles (Lopez et al. 2002; Andujar-Ortiz et al. 2009). Therefore, the first step in the work was to select the most suitable polymer, among Tenax and Lichrolut to be used in the RATD for the *in vivo* aroma release experiments with model

wine derived beverages. In order to test these two types of materials, dynamic headspace analysis was used as experimental approach in trying to mimic, as much as possible, the dynamic working conditions of the RATD during the *in vivo* aroma analysis, avoiding the use of human subject in this first step of the work, which is linked to some experimental drawbacks (inter-individual differences, fatigue, limited number of experiments, etc). For this type of analysis, aroma compounds contained in a model wine were flushed with a N_2 stream and trapped in the corresponding polymer. **Figure 2** shows the comparison between both types of traps. As it can be seen, both trapping materials provided in general, the same extraction yield for most of the aroma compounds. However, β-ionone and β-phenylethanol were more significantly recovered using Tenax.

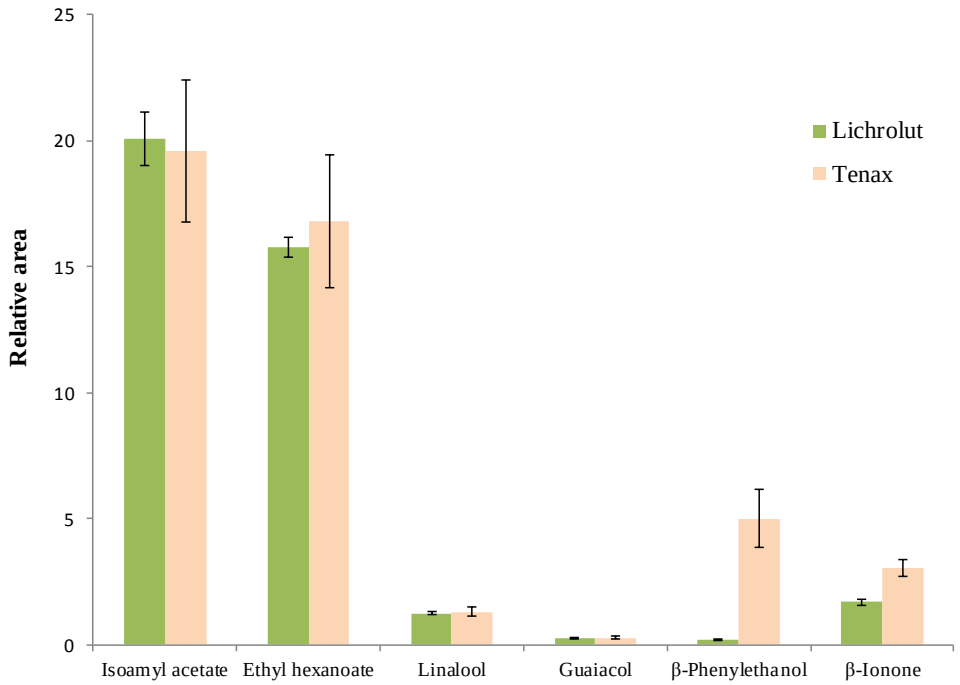


Figure 2. Comparison of the extraction performance (relative peak areas) of the two polymeric traps (Lichrolut and Tenax) employed for the extraction of aroma compounds in a model wine beverage using dynamic headspace analysis. Asterisks denote significant differences among samples (P > 0.05).

Regarding the extraction yield of different types of aroma compounds by using the same adsorbent polymer, it is important to consider that not only the affinity of the compounds for the adsorbent material, but also their response factors (RFs) in the MS (see Dynamic headspace-GCMS analysis in Material and Methods for RFs values) can also affect. In this sense, the esters isoamyl acetate and ethyl hexanoate with the highest RFs also showed the greatest extraction yields independently of the employed polymer.

On the contrary, guaiacol showed the lowest recovery no matter the polymer employed for the trapping. This was not because of its poor signal in the MS detector, since other compounds with lower RFs than guaicol (e.g. linalool, β-phenylethanol) were however, more recovered. Previous works have also shown a low recovery of this compound during the SPE analysis of wine volatiles using Lichrolut (Lopez et al. 2002; Andujar-Ortiz et al. 2009). Similarly, β-ionone was very little recovered with either of the two traps, while it exhibited quite high RFs in the MS. In general, the aroma compounds with the lowest log P values (**Table 1**), like guaicol, were the least recovered. This contrasts with results from Aznar and co-workers (Aznar et al. 2004) who showed a decrease in the headspace of ethanolic solutions with an increase in the log P value until log *P* = 3. This disagreement could be due to the different methodology employed in the above mentioned study (static headspace) compared to this one (dynamic headspace). In dynamic conditions, Tsachaki and collaborators (Tsachaki et al. 2005) did not find a clear relationship between log P and headspace release, which they attributed to the surface active properties of ethanol, which is involved in the so-called Marangoni effect.

The *inter*- and *intra*-trap variations during the extraction using both types of polymers were also determined (**Table 3**). The intra-trap variation (n=5) was lower than 10 % for most of the compounds using both types of trapping materials; however, it was higher (14.7 %) for β-phenylethanol by using Lichrolut and for guaicol (13.5 %) using Tenax. These two compounds also had the lowest log P values, as it was said before.

Table 3. Intra- and Inter-trap variation using Tenax and Lichrolut polymers during the dynamic headspace analysis (purge and trap) of the model wines.

	Intra-Trap RSD (%)		*Inter*-Trap RSD (%)	
	Lichrolut	Tenax	Lichrolut	Tenax
Isoamyl acetate	5.19	8.75	19.22	7.28
Ethyl hexanoate	2.49	8.83	22.00	8.24
Linalool	4.95	8.05	22.21	5.53
Guaiacol	9.54	13.54	7.47	14.58
β-Phenylethanol	14.70	3.86	13.27	6.30
β-Ionone	7.32	7.99	29.59	4.01

RSD: Relative standard deviation (%); *n*=5 in both experiments.

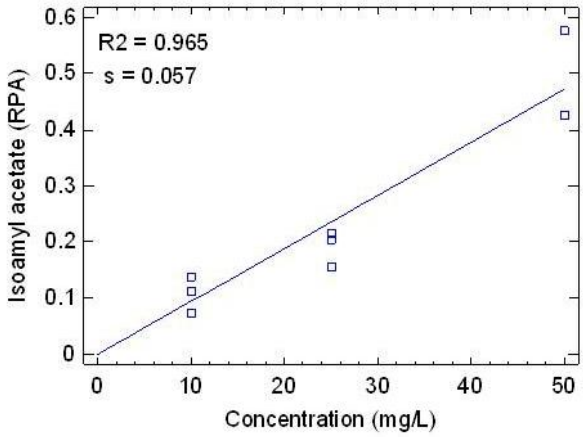
Many more differences between the two types of polymers were found when comparing the inter-trap variability (n = 5). Herein, Lichrolut clearly showed the highest variation, while Tenax trap in general kept, very similar values to those calculated for the intra-trap variation (< 10%) for all the aroma compounds except guaiacol. Therefore, taking into consideration the good recovery for most of the volatile compounds of interest and the lower inter- and intra-trap variability, we decided to use Tenax for the *in vivo* retronasal trapping device.

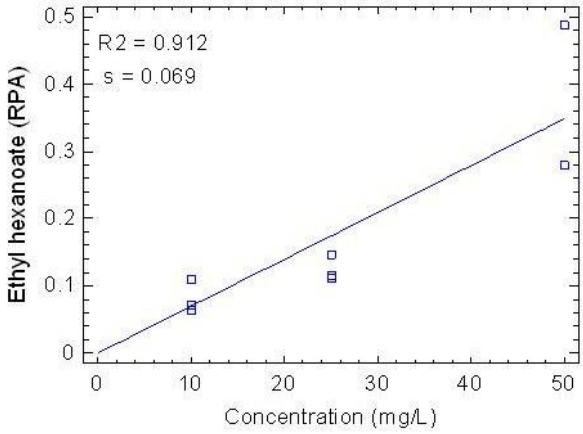
Analytical performance of the Retronasal Aroma Trapping Device (RATD)

Once the trapping material was selected, the analytical performance of the RATD in real experimental conditions (drinking conditions) using human assessors was tested. For these experiments the model wine beverage MWB-1 was used. To improve the acceptability and pleasantness of the beverages for the assessors, only an aroma mixture composed of three aroma compounds (ethyl hexanoate, isoamyl acetate and linalool) at the same concentration (25 mg L^{-1}) was used to aromatize the wine based beverages for all the *in vivo* experiments.

Dynamic linear range of the RATD

The dynamic linear range of the RATD was calculated for the three compounds of interest. To do so, the same beverage (MWB-1) was prepared spiking different concentrations of the mixture of aroma compounds covering a wide range of concentrations (0, 10, 25 and 50 mg L^{-1}). Following previous studies (Buettner and Schieberle 2000) and in order to avoid the inter-individual differences, the beverage was consumed by the same panelist following the *in vivo* aroma release procedure in two different sessions as previously described. The regression models calculated for the compounds of interest are depicted in **figure 3.**





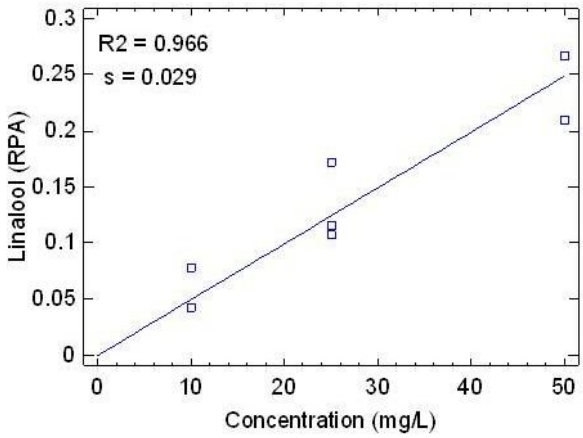


Figure 3. Regression models calculated for the three aroma compounds after the consumption of a model wine beverage with different aroma concentrations by using the RATD-GCMS analysis. P values for the calculated modes were: 0.00001, 0.0001 and 0.00001 for isoamyl acetate, ethyl hexanoate and linalool respectively.

A lack of fit test was also applied to determine whether the calculated model was adequate for the experimental data. As it can be seen, a clear linear relationship between the amount of aroma compounds (relative peak areas) in the exhaled breath of the individual and the concentration of aroma compounds in the beverages was obtained. The linear models showed determination coefficients higher than 90 % for the three essayed compounds: ethyl hexanoate (R^2 = 0.911), isoamyl acetate (R^2 = 0.964) and linalool (R^2 = 0.966) and adequate values of residual standard deviation (s) in the concentration range between 0 and 50 mg L^{-1}, showing the adequacy of the RATD to study aroma release in this type of wine samples.

Inter-individual differences on aroma release patterns

The variability on the total aroma released between panelists equally trained in the same consumption procedure and using the optimized RATD conditions was also determined. Three panelists were instructed to drink the same type of beverage (MWB-1) following the previously described drinking procedure. The graphs showing the aroma release during consumption are presented in **figure 4**.

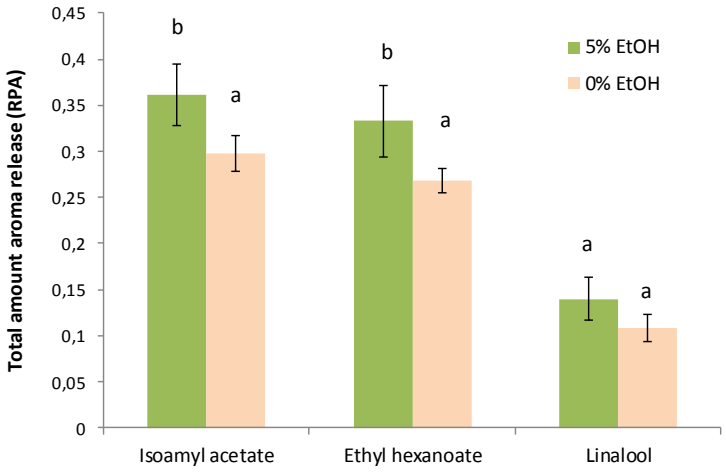


Figure 4. Total aroma release (relative peak area) during the consumption of MWB-1 by three trained assessors determined by RATD-GCMS analysis. Different letters across the different assessors denotes statistical differences (P < 0.05) after the application of the LSD test.

As it can be seen, in spite of the training, the panelists exhibited significant differences on the total aroma release patterns (expressed as relative peak area) during drinking. Assessor #3 exhibited the highest aroma release for isoamyl acetate and ethyl hexanoate, while assessors #1 and #2 did not show significant differences for the release

of isoamyl acetate, and they also slightly differed on the release of ethyl hexanoate. However, no significant differences in the release of linalool were found between the panelists. Inter-individual differences in the aroma release patterns during drinking were previously observed because of the differences in physiological variables (mouth volumes, swallowing, breathing patterns, etc. (Buettner et al. 2001; Buettner et al. 2002; Weel et al. 2003; Weel et al. 2004; Deleris et al. 2011)). In addition to these physiological factors, these results clearly showed that the type of aroma compound (physicochemical characteristics), also has a significant influence on the aroma release, which is in agreement with previous works performed in other food matrices and with other methodologies to monitor aroma release (Saint-Eve et al. 2009; Deleris et al. 2011; Deleris et al. 2013).

Impact of ethanol and sugar on in vivo aroma release during the consumption of model wine beverages

Some works performed in *in vitro* conditions (static or dynamic headspace conditions) have shown that wine matrix composition might play an important role on the interaction with aroma compounds (Dufour and Bayonove 1999; Dufour and Bayonove 1999; Dufour and Sauvaitre 2000; Pozo Bayón and Reineccius 2009; Robinson et al. 2009; Rodriguez-Bencomo et al. 2011). Even so, these interactions might affect the sensory characteristics of the wines (Jones et al. 2008; Saenz-Navajas et al. 2010). Therefore, any attempt to formulate any type of wine base beverage should determine whether the composition might be considered as an important variable when determining aroma release during real drinking conditions. Thus, once the validity of the RATD to determine the aroma released during the consumption of these types of beverages was proven, four model wine beverages were formulated following the recipes previously described differing in two main ingredients; the presence or absence of ethanol and sugar (**Table 2**). Despite the fact that both ingredients have been described to have a large influence on aroma release from wines in static headspace aroma analysis (Escalona et al. 1999; Rodriguez-Bencomo et al. 2002; Aznar et al. 2004), their influence during the *in vivo* consumption of wine or wine based beverages have not been currently explored. To determine solely the effect of matrix composition on the aroma release, whilst avoiding the inter-individual differences previously shown, each of the four model wine beverages (MWB-1, MWB-2, MWB-3 and MWB-4) were

consumed by a single assessor in two different sessions following the procedure previously described using the RATD.

Aroma release data were submitted to a two-way factorial ANOVA to determine the effect of the two ingredients. Results from the test did not show a significant effect of adding 15 g L^{-1} of sucrose into the beverage. However, a significant effect of ethanol was shown on the release of isoamyl acetate and ethyl hexanoate. The absence of a significant effect of sucrose on the aroma release is in agreement with the results from Weell and co-workers (Weel et al. 2003), who did not observe differences in aroma release during the consumption of a 10 g L^{-1} sucrose added to a lemon-lime type beverage compared with the reference beverage without the sweetener. In addition, Saint-Eve and collaborators (Saint-Eve et al. 2009) did not find a significant influence in the addition of 1g L^{-1} sucrose on the aroma release during the consumption of mint flavoured beverages either. However, other scientific works performed in static conditions, have pointed out some sucrose-flavour physicochemical interactions, although in general, these works were performed with higher sucrose concentrations (from 20 to 60 g L^{-1}), and have been attributed to a *salting out* effect of sucrose, whereby sucrose interacts with water, increasing the concentration of flavour compounds in the remaining free water (Nahon et al. 1998; Friel et al. 2000; Hansson et al. 2001). Therefore, considering that the concentration of sucrose fit within the concentrations normally used in these beverages (5-15 g L^{-1}), it could be concluded that the concentration of sugar does not have a significant effect on aroma release during drinking. Nonetheless, it is important to highlight that this conclusion might not be true for the aroma perception, since perceptual differences are also linked to psychophysical effects(Weel et al. 2003).

Regarding the influence of ethanol on aroma release, an LSD test was applied to the data in order to determine the magnitude of the observed effect**. Figure 5** shows these results in which the average aroma release during the consumption of the model wine beverages with and without ethanol (independently of the sugar content, as it did not significantly affect aroma release) are shown.

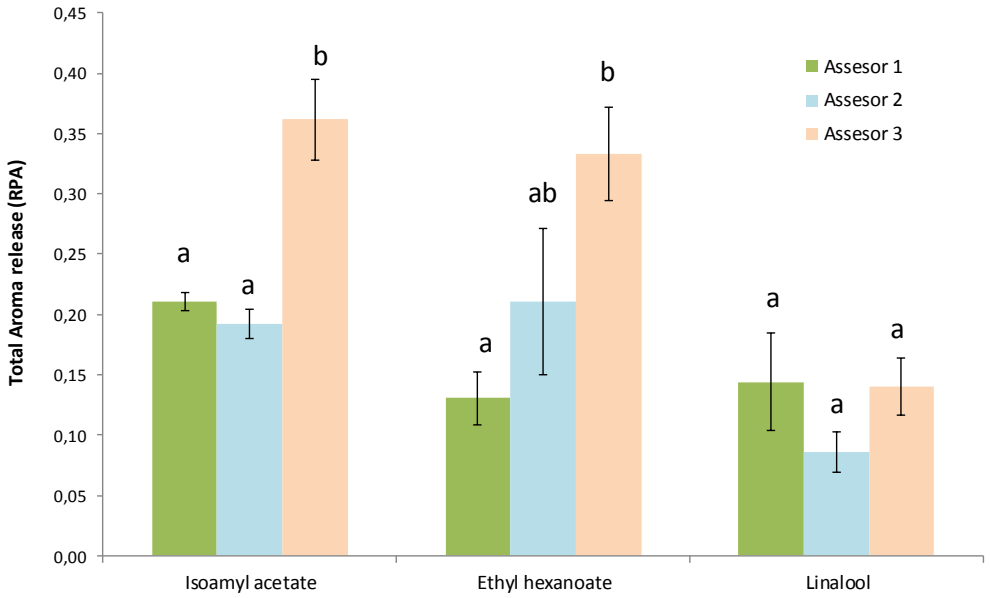


Figure 5. Influences of ethanol on the aroma release during the consumption of model wine derived beverages using RATD-GCMS analysis. Different letters across the different wine samples denotes statistical differences ($P < 0.05$) after the application of the LSD test.

As it can be seen, the presence of 5 mL L^{-1} ethanol increased the aroma release during consumption, above 18 % for isoamyl acetate and 22 % in the case of ethyl hexanoate. For linalool, the average aroma release values were also higher in the model wines with ethanol, although they were not statistically significant. However, these results showed the same trend; an enhancement of aroma release in presence of ethanol. Recently, Clark and co-authors (Clark et al. 2011) also showed by using *in vivo*-API-MS a similar rise in the release of three targeted aroma compounds during the *in vivo* consumption of flavoured model beers with an increase in the ethanol content from 0 to 4.5 mL L^{-1}. Contrarily, most of the studies dealing with the effect of ethanol on aroma release performed in static conditions have shown a reduction in the aroma released into the headspace. This effect has been explained as consequence of the higher solubility of aroma compounds due to an increase in the ethanol concentration (Aznar et al. 2004; Aprea et al. 2007). This fact highlights the idea that static headspace techniques used to monitor aroma release do not provide the same conclusions as the works performed *in vivo*, independently of the methodology used to monitor aroma release during consumption (on-line employing API-MS or off-line using the RATD as in the present work). Differences between *in vitro* and *in vivo* studies might be due to the effect of all

the oro-physiological parameters (breathing and swallowing patterns, saliva, mucus, etc) involved in the *in vivo* delivery of aroma compounds during drinking.

To explain the enhancement effect of ethanol on aroma release in the *in vivo* studies, different hypotheses have been proposed (Clark et al. 2011). The first one has been associated to the change that ethanol might induce in surface tension affecting the distribution of the liquid in the mouth and pharynx during consumption, allowing the sample to better spread out and favoring the formation of a larger surface in the pharynx for volatile release. Another effect of ethanol could be linked to its capacity to increase the solubility of aroma compounds in the aqueous coating of the mouth and throat preventing losses and/or increasing the amount of volatile compounds at the gas-liquid interface, which might enhance aroma release. Finally, the so-called Marangoni effect (Hosoi and Bush 2001), could also be involved. In this case, the evaporation of ethanol in the gas-liquid interface of the mouth and throat might create a streaming of new ethanol molecules and volatile compounds to replenish those released, which might increase the amount of aroma released(Tsachaki et al. 2005).

Conclusions

In summary, the RATD and the consumption procedure optimised in this work, allows in a simple, convenient, and precise way, the determination of the impact of matrix components on aroma release during real drinking conditions of model wine derived beverages. The impact of ethanol, increasing the total amount of aroma release during drinking has been proven, which should be taken into consideration during the formulation of new types of wine derived beverages; however, the impact of this fact on the sensory characteristics of the beverage should be achieved in future works. In addition, results of this work have shown the importance of collecting real *in vivo* data to extract truthful conclusions about the effect of beverages components on aroma release during consumption, highlighting the idea that besides its composition, the overall perceived flavour of a food or beverage is mainly impacted by the way in which volatile aroma compounds are released in the mouth and transported to the olfactory receptors in the nose during consumption. However, new experiments involving a higher number of assessors and sensory test should be performed in order to corroborate the effect of these two ingredients on aroma release and their impact on aroma perception.

References

Andujar-Ortiz, I., M. V. Moreno-Arribas, et al. (2009). "Analytical performance of three commonly used extraction methods for the gas chromatography-mass spectrometry analysis of wine volatile compounds." Journal of Chromatography A 1216(43): 7351-7357.

Aprea, E., F. Biasioli, et al. (2007). "PTR-MS study of esters in water and water/ethanol solutions: Fragmentation patterns and partition coefficients." International Journal of Mass Spectrometry 262(1-2): 114-121.

Aznar, M., M. Tsachaki, et al. (2004). "Headspace analysis of volatile organic compounds from ethanolic systems by direct APCI-MS." International Journal of Mass Spectrometry 239(1): 17-25.

Buettner, A., A. Beer, et al. (2001). "Observation of the swallowing process by application of videofluoroscopy and real-time magnetic resonance imaging-consequences for retronasal aroma stimulation." Chemical Senses 26(9): 1211-1219.

Buettner, A., A. Beer, et al. (2002). "Physiological and analytical studies on flavor perception dynamics as induced by the eating and swallowing process." Food Quality and Preference 13(7-8): 497-504.

Buettner, A. and P. Schieberle (2000). "Exhaled odorant measurement (EXOM) - A new approach to quantify the degree of in-mouth release of food aroma compounds." Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology 33(8): 553-559.

Clark, R., R. Linforth, et al. (2011). "Effects of Ethanol, Carbonation and Hop Acids on Volatile Delivery in a Model Beer System." Journal of the Institute of Brewing 117(1): 74-81.

Delahunty, C. M., J. R. Piggott, et al. (1996). "Comparison of dynamic flavour release from hard cheeses and analysis of headspace volatiles from the mouth with flavour perception during consumption." Journal of the Science of Food and Agriculture 71(3): 273-281.

Deleris, I., A. Saint-Eve, et al. (2011). "Impact of Swallowing on the Dynamics of Aroma Release and Perception during the Consumption of Alcoholic Beverages." Chemical Senses 36(8): 701-713.

Deleris, I., A. Saint-Eve, et al. (2013). "Comparison of direct mass spectrometry methods for the on-line analysis of volatile compounds in foods." Journal of Mass Spectrometry 48(5): 594-607.

Dufour, C. and C. Bayonove (1999). "Interactions between Wine Polyphenols and Aroma Substances. An Insight at the Molecular Level." Journal of Agricultural and Food Chemistry 47(2): 678-684.

Dufour, C. and C. L. Bayonove (1999). "Influence of wine structurally different polysaccharides on the volatility of aroma substances in a model system." Journal of Agricultural and Food Chemistry 47(2): 671-677.

Dufour, C. and I. Sauvaitre (2000). "Interactions between anthocyanins and aroma substances in a model system. Effect on the flavor of grape-derived beverages." Journal of Agricultural and Food Chemistry 48(5): 1784-1788.

Escalona, H., J. R. Piggott, et al. (1999). "Effect of ethanol strength on the volatility of higher alcohols and aldehydes." Italian Journal of Food Science 11(3): 241-248.

Friel, E. N., R. S. T. Linforth, et al. (2000). "An empirical model to predict the headspace concentration of volatile compounds above solutions containing sucrose." Food Chemistry 71(3): 309-317.

Genovese, A., P. Piombino, et al. (2009). "Simulation of retronasal aroma of white and red wine in a model mouth system. Investigating the influence of saliva on volatile compound concentrations." Food Chemistry 114(1): 100-107.

Hansson, A., J. Andersson, et al. (2001). "Effect of changes in pH on the release of flavour compounds from a soft drink-related model system." Food Chemistry 74(4): 429-435.

Hosoi, A. E. and J. W. M. Bush (2001). "Evaporative instabilities in climbing films." Journal of Fluid Mechanics 442: 217-239.

Jones, P. R., R. Gawel, et al. (2008). "The influence of interactions between major white wine components on the aroma, flavour and texture of model white wine." Food Quality and Preference 19(6): 596-607.

Lasekan, O., A. Buettner, et al. (2009). "Investigation of the retronasal perception of palm wine (Elaeis guineensis) aroma by application of sensory analysis and exhaled odorant measurement (EOM)." African Journal of Food, Agriculture, Nutrition and Development 9(2): 793-813.

Lindinger, W., A. Hansel, et al. (1998). "Proton-transfer-reaction mass spectrometry (PTR-MS): on-line monitoring of volatile organic compounds at pptv levels." Chemical Society Reviews 27(5): 347-354.

Lopez, R., M. Aznar, et al. (2002). "Determination of minor and trace volatile compounds in wine by solid-phase extraction and gas chromatography with mass spectrometric detection." Journal of Chromatography A 966(1-2): 167-177.

Margomenou, L., L. Birkmyre, et al. (2000). "Optimisation and validation of the "strathclyde simulated mouth" for beverage flavour research." Journal of the Institute of Brewing 106(2): 101-105.
Munoz-Gonzalez, C., J. J. Rodriguez-Bencomo, et al. (2011). "Beyond the characterization of wine aroma compounds: looking for analytical approaches in trying to understand aroma perception during wine consumption." Analytical and Bioanalytical Chemistry 401(5): 1497-1512.

Nahon, D. F., P. Koren, et al. (1998). "Flavor release from mixtures of sodium cyclamate, sucrose, and an orange aroma." Journal of Agricultural and Food Chemistry 46(12): 4963-4968.

Poinot, P., G. Arvisenet, et al. (2013). "How can aroma-related cross-modal interactions be analysed? A review of current methodologies." Food Quality and Preference 28(1): 304-316.

Pozo Bayón, M. A. and G. Reineccius (2009). Interactions Between Wine Matrix Macro-Components and Aroma Compounds Wine Chemistry and Biochemistry M. V. Moreno-Arribas and M. C. Polo. New York, Springer New York II: 417-435

Robinson, A. L., S. E. Ebeler, et al. (2009). "Interactions between Wine Volatile Compounds and Grape and Wine Matrix Components Influence Aroma Compound Headspace Partitioning." Journal of Agricultural and Food Chemistry 57(21): 10313-10322.

Rodriguez-Bencomo, J. J., J. E. Conde, et al. (2002). "Determination of esters in dry and sweet white wines by headspace solid-phase microextraction and gas chromatography." Journal of Chromatography A 963(1-2): 213-223.

Rodriguez-Bencomo, J. J., C. Munoz-Gonzalez, et al. (2011). "Assessment of the effect of the non-volatile wine matrix on the volatility of typical wine aroma compounds by headspace solid phase microextraction/gas chromatography analysis." Journal of the Science of Food and Agriculture 91(13): 2484-2494.

Saenz-Navajas, M. P., E. Campo, et al. (2010). "Effects of the Nonvolatile Matrix on the Aroma Perception of Wine." Journal of Agricultural and Food Chemistry 58(9): 5574-5585.

Saint-Eve, A., I. Deleris, et al. (2009). "Influence of Composition(CO2 and Sugar) on Aroma Release and Perception of Mint-Flavored Carbonated Beverages." Journal of Agricultural and Food Chemistry 57(13): 5891-5898.

Smyth, H. and D. Cozzoino (2013). "Instrumental Methods (Spectroscopy, Electronic Nose, and Tongue) As Tools To Predict Taste and Aroma in Beverages: Advantages and Limitations." Chemical Reviews 113(3): 1429-1440.

Taylor, A. J. and L. R.S. (2000). Techniques for measuring volatile release in vivo during consumption of food. Flavor Release. R. D.D. and T. A.J. Washington D.C., American Chemical Society. ACS Symposium series 763: 8-21.

Tsachaki, M., R. S. T. Linforth, et al. (2005). "Dynamic headspace analysis of the release of volatile organic compounds from ethanolic systems by direct APCI-MS." Journal of Agricultural and Food Chemistry 53(21): 8328-8333.
Weel, K. G. C., A. E. M. Boelrijk, et al. (2003). "A protocol for measurement of in vivo aroma release from beverages." Journal of Food Science 68(3): 1123-1128.

Weel, K. G. C., A. E. M. Boelrijk, et al. (2004). "New device to simulate swallowing and in vivo aroma release in the throat from liquid and semiliquid food systems." Journal of Agricultural and Food Chemistry 52(21): 6564-6571.

Publicación 3. Impacto de la composición de la matriz no volátil del vino en la liberación *in vivo* del aroma de vinos.

Impact of the nonvolatile wine matrix composition on the in vivo aroma release from wines

Carolina Muñoz-González; Pedro J. Martín-Álvarez; M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón

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Abstract

The impact of the nonvolatile wine matrix composition on the retronasal aroma release of four volatile compounds added to different types of wines has been evaluated. For this purpose, a tailor made retronasal aroma trapping device (RATD) was used to entrap the exhaled breath of six panelists previously trained in a specific consumption procedure. Five wines of different composition (white wine, sparkling white wine, young red wine, aged red wine and a sweet wine) were evaluated. Prior to the evaluation, with the exception of the sweet wine, the wines were adjusted to the same ethanol content and aromatized with a mixture of four target volatile compounds. Aroma release data were submitted to multivariate statistical analysis in order to relate wine chemical composition and aroma release during wine drinking. Results showed inter-individual differences and a clustering of panelists among lower and higher aroma releasers, which was in agreement to the differences in their breathing capacity. A significant influence of the matrix composition in the low aroma releasers group during wine consumption was observed. The consumption of red wines provoked a significantly higher aroma release than the consumption of white and sweet wines. From the chemical composition determined in the wine samples (pH, total acidity, total polyphenols, neutral polysaccharides, residual sugar and nitrogenous compounds), the amount of total polyphenols was better correlated with the observed effect.

Introduction

The hedonic behavior behind wine consumption is greatly influenced by wine aroma, which is one of the most outstanding characteristics in explaining wine quality. Wine aroma is composed of hundreds of volatile compounds which depending on their concentration and chemical structure will have a higher or lower impact on the overall flavor profile of a wine (Polaskova et al. 2008; Munoz-Gonzalez et al. 2011) The characterization of these compounds and the elucidation of their sensory relevance for wine aroma have been the focus of many interesting works (Guth 1997; Kotseridis and Baumes 2000; Escudero et al. 2007) and taking into account the magnitude of this task, it is likely these works will continue in the future.

However, we already know that only the characterization of the overall flavor composition of a food (for example: the headspace profile) does not directly correlate with the perceived sensations during consumption (Buettner et al. 2002). The process of aroma release during food consumption is a sequential process, which starts when the food is smelt (orthonasal aroma) and continues during the processing of the food in the mouth. Within the oral cavity, volatile compounds are released from the food and from here breathing flow carries them to the olfactory region where they are perceived. This is a dynamic and complex process known as retronasal aroma, in which not only the physicochemical characteristics of the compounds, but other physiological factors such as breathing flow, the presence of saliva and tongue movements are also involved (Linforth and Taylor 2000; Buettner et al. 2002). For liquid foods, such as wine, although the processing in the mouth could not be as critical, there are some factors such as the formation of a coating on the pharyngeal mucosa, the flow rates, temperature, etc., that should be considered as important aspects for modulating the aroma released available for the olfactory receptors during consumption (Linforth and Taylor 2000; Buettner et al. 2001; Weel et al. 2004; Deleris et al. 2011).

In spite that retronasal aroma is directly related to flavor perception and it is a key modulator for food consumption and food preferences, there are not many scientific works directed towards understanding aroma release during wine consumption. This type of study would need new analytical approaches based on monitoring aroma release during drinking (Munoz-Gonzalez et al. 2011). One possible approach is the use of artificial devices to simulate the drinking process. Following this *in vitro* approach,

Genovese and collaborators (Genovese et al. 2009) studied the influence of saliva on wine aroma release. Although, undoubtedly, the use of artificial devices has considerable advantages (better control of the variables of the study, many experimental repetitions, no requirements of human subjects avoiding ethical considerations, etc), they cannot mimic the real situation during wine consumption, therefore, they do not take into consideration all the physiological factors involved in aroma release (such as swallowing), making their correlation with sensory perception difficult. Other approaches to monitor aroma release during *in vivo* food consumption, such as the use of real time monitoring (breath by breath analysis) using mass spectrometric techniques such as atmospheric pressure ionization or proton transfer reaction mass spectrometric techniques (API-MS and PTR-MS respectively) (Lindinger et al. 1998; Taylor and R.S. 2000), or the use of aroma trapping devices of exhaled breath during consumption (Linforth et al. 1994; Delahunty et al. 1996; Buettner and Schieberle 2000) which are often employed to study aroma release in many types of liquid and solid foods, have not been often applied in the wine field. Only Lasekan and co-workers (Lasekan et al. 2009) applied an exhaled odor trapping device to investigate the aroma compounds released during the consumption of palm wine.

On the other hand, in recent years, the influence of the nonvolatile matrix composition has been highlighted as an outstanding factor influencing wine aroma release (Dufour and Bayonove 1999; Dufour and Bayonove 1999; Dufour and Sauvaitre 2000; Pozo Bayón and Reineccius 2009; Robinson et al. 2009; Saenz-Navajas et al. 2010; Rodriguez-Bencomo et al. 2011) Using specific wine non-volatile components (Dufour and Bayonove 1999; Dufour and Bayonove 1999; Dufour and Sauvaitre 2000; Robinson et al. 2009) or the whole wine matrix composition (Saenz-Navajas et al. 2010; Rodriguez-Bencomo et al. 2011) it has been possible to determine the interaction effect between wine compounds and specific wine volatiles. Even the effect of these interactions on the sensory characteristics of wines has been shown (Jones et al. 2008; Saenz-Navajas et al. 2010). Most of these analytical studies have been carried out in static conditions, which, although very valuable, do not represent the retronasal aroma delivery of volatiles during a real wine consumption situation.

Therefore, the objective of this work was to determine the impact of the non-volatile wine matrix composition on the *in vivo* aroma release during the consumption of different types of wines. To achieve the objective of this work, a tailor made

retronasal aroma trapping device (RATD) was used to trap the exhaled breath of six panelists previously trained in a specific consumption procedure. Five wines of different composition were aromatized with a mixture of volatile compounds at the same concentration and the aroma release data were submitted to multivariate statistical analysis in order to relate wine chemical composition and aroma release during wine drinking.

Material and methods

Wine samples

Five commercial Spanish wines representative of different winemaking technologies and with different matrix compositions were selected for this study: a young Verdejo white wine (**WH-W**), a young Tempranillo red wine (**YR-W**), a 4-year old (16 months in oak barrels) Tempranillo red wine (**AR-W**), a Cava white wine (Spanish sparkling wine manufactured by the traditional method) (**SP-W**) and a sweet biologically aged wine made from Pedro Ximénez grapes (**SW-W**).

In order to minimize the effect of ethanol on the volatility of aroma compounds, all the wines, except the sweet wine that was kept at its initial concentration (15% v/v) were adjusted to the same ethanol content (13.5% v/v). All the wines were spiked with four *food-grade* aroma compounds from Sigma-Aldrich (Steinheim, Germany) characterized by presenting different physicochemical properties (**Table 1**).

Table 1. Main physicochemical characteristics of the aroma compounds used in the study of aroma release during the consumption of different types of wine matrices.

Compound	Chemical group	Molecular weight (g/ mol)	Log *P*[a]	Boiling point (ºC)	Solubility[b] (mg/L)
Isoamyl acetate	Ester	130.2	2.3	134.9	1070.0
Ethyl hexanoate	Ester	144.1	2.8	167.0	308.7
Linalool	Terpene	152.2	3.4	204.1	683.7
β-phenylethanol	Alcohol	122.2	1.6	224.9	21990.0

[a] Hydrophobic constant and [b] solubility in water at 25ºC estimated using molecular modeling software EPI Suite (U.S. EPA 2000-2007).

To do that, four independently aroma stock solutions in ethanol absolute were prepared and from here, each aroma compound was added to the wines to obtain a final

concentration of 50 mg/L. The initial concentration of these aroma compounds in the original wines was also previously determined in the five types of wines (Rodríguez-Bencomo et al., 2011) showing that endogenous aroma represent a 5% of the aroma added to the wines; except for *β*-phenylethanol, which concentration corresponded to 15%. The considerably lower amount of these compounds in the original wines compared to the higher amount of the exogenously added aroma should not interfere on the solubility or volatility properties of the added aroma compounds as has been previously shown (Athes et al., 2004).

Retronasal Aroma Trapping Device (RATD)

A tailor made retronasal trapping device (RATD) was employed for this study. This glass device allowed the trapping of exhaled breath after wine consumption into a Tenax trap thanks to a nosepiece coupled to a hollow tube in which the trap was fitted. A vacuum pump connected to a rotameter allowed a steady flow through the trap. Finally, a flowmeter allowed us to know the exact flow through the trap. In preliminary experiments, an optimization and validation of the effectiveness of this device for the purposes of this study was performed. The retention time, ion of quantification, the range of concentration assayed for each volatile compound and regression lines, together with the values of the residual standard deviation (*s*) and the determination coefficient (R^2), which are estimators of the adequacy of the regression models, are presented in **Table 2**. In addition, the inter- and intra-traps variability is presented. Adequate relative standard deviation between analysis, very low values of *s* and satisfactory values for the regression coefficients were obtained for the assayed compounds: isoamyl acetate (R^2 = 0.964), ethyl hexanoate (R^2 = 0.991), linalool (R^2 = 0.985) and *β*-phenylethanol (R^2 = 0.978).

Retronasal aroma trapping procedure during wine consumption

Six volunteers (2 males and 4 females) between 26-34 years old previously trained in the retronasal aroma trapping procedure participated in this study. They were instructed not to eat, drink or smoke 2 h before the experiments. They had no known illnesses and had self-reported normal olfactory and gustatory functions. Before each experiment, the panelists had to clean their mouths and rinse with a bicarbonate solution. The monitoring of the oral cavity of the panelists for the four compounds of interest was performed before each analysis.

Table 2. Chromatographic and regression parameters ($y = a + bx$) determined for the added aroma compounds in the breathing trapping experiments.

			Linear regression[c]					Repeatability[d]	
	RT[a]	**ion Q[b]**	**linear range (mg/L)**	**a**	**b**	***s***	***R²***	**Inter-cartridge RSD (%)**	**Intra-cartridge RSD (%)**
Isoamyl acetate	11.42	43	25-100	0.2209	0.0126	0.0930	96.4	13.3	6.8
Ethyl hexanoate	13.32	88	0-100	-	0.0142	0.0982	99.1	23.0	14.9
Linalool	18.53	71	0-100	-	0.0053	0.0487	98.5	11.6	11.8
β-phenylethanol	24.35	91	0-90	-	0.0002	0.0018	97.8	19.0	19.5

[a] RT = Retention time (minutes); [b] ion Q = quantification ion; [c] Linear regression parameters = intercept (a) , slope (b), residual standard deviation (*s*) and determination coefficient (R^2); [d] = Repeatability is expressed as an average of 5 assays performed in the same conditions.

The drinking procedure consisted in mainly two steps. In the first one, 20 mL of wine contained in a syringe were provided to the panelist, who had to keep it in the mouth for 10 seconds with the lips closed. After this time, the panelist had to swallow and breath normally through the nose into the glass nosepiece for 20 more seconds. During the experiment, a relatively small amount of wine, typically consumed with a meal (100 mL) was provided. Two repetitions of the same wine were performed on the same day but on different seasons (leaving at least 2 hours between replications). The same procedure was followed with the original control wines (without aroma added), so that we could find out the amount of aroma initially present in the wines that could be trapped with this device. These results were used to correct the aroma release data.

Analysis of volatile compounds

The aroma compounds from the expiration of breath by the nose and trapped onto the Tenax cartridges were desorbed with 6 mL of a hexane/diethyl ether solution (1:1). Thirty microliters of an internal standard (3-octanol) were added, and the sample was then concentrated using a nitrogen stream to a final volume of 200 µL and analyzed in the GC-MS. A volume of 8 µL of the concentrated breath extract was injected in a cool injection system unit (CIS), (Gerstel, Mülheim an der Ruhr, Germany) in the solvent vent mode. The injector temperature ranged from -80 ºC to 270 °C.

The identification of volatile compounds was carried out with a Gas Chromatograph Agilent 6890N coupled to a quadrupole Mass Detector Agilent 5973. After injection on the CIS, volatile compounds were separated on a Supra-Wax polar capillary column (60 m × 0.25 mm i.d. × 0.50 µm film thickness) from Konik (Barcelona, Spain). Helium was used as the carrier gas at a flow rate of 1 mL/min. The oven temperature was initially held at 50 ºC for 2 min, then increased at 8 ºC/min to 240 ºC and held for 15 min.

For the MS system (Agilent 5973N), the temperatures of the transfer line, quadrupole and ion source were 270, 150 and 230 ºC respectively. Electron impact mass spectra were recorded at 70 eV ionization voltages and the ionization current was 10 µA. The acquisitions were performed in Scan (from 35 to 350 amu) and SIM modes. The identification of compounds was based on the comparison of retention times and mass spectra. The mass spectra were compared with those from NIST 2.0 database. Relative peak areas (RPAs) were obtained by calculating the relative peak area in

relation to that of the internal standard. The use of RPAs data to express aroma release was sufficient for this type of analysis as the aim of the work was to compare the extent of aroma release between wine samples.

Wine matrix composition

Nitrogen compounds (total nitrogen, free amino acids and peptides), neutral polysaccharides and residual sugars, total polyphenols, total acidity and pH were determined in the five types of wines following the procedures previously described in Rodriguez-Bencomo et al., (2011).

Statistical analysis

The statistical methods used for data analysis were: linear regressions to establish the regression parameters for each aroma compound released after wine drinking and the lack of fit test to judge the adequacy of the linear models; cluster analysis (the square Euclidean distance was taken as a measure of the proximity between two samples and Ward's method was used as linkage rule) to check the grouping of panelists depending on their aroma release performance; one-way ANOVA to test the influence of matrix composition on aroma release and to test compositional differences between wines; least significant difference (LSD) test for mean comparison; principal component analysis (PCA) to examine the relationship between compositional parameters and wine matrices; and correlation analysis to determine the existence of correlations between each of the compositional variables and the aroma release data of the four aroma compounds. The STATISTICA program for Windows version 7.1 was used for data processing (StatSoft, Inc., 2005, www.statsoft.com).

Results and discussion

In vivo aroma release from wines

Different published works have shown that wine matrix might play an important role on aroma release in static headspace conditions (Aronson and Ebeler 2004; Pineau et al. 2007; Pozo Bayón and Reineccius 2009; Robinson et al. 2009; Saenz-Navajas et al. 2010; Rodriguez-Bencomo et al. 2011). However, the relevance of this effect on a real wine consumption situation has not been evaluated so far. In this work, we have focused on the *in vivo* release (during wine consumption) of some target aroma

compounds added to five wines with different matrix composition. We monitored, the so-called 'expiration breath', since it might be a good representation of the aroma compounds which interact with the olfactory system (Buettner and Schieberle 2000). The type of aroma compounds (isoamyl acetate, ethyl hexanoate, linalool and *β*-phenylethanol) represented a wide range of physicochemical properties and the concentration was selected in order to make an easy-to-drink wine, preventing the possible tiredness and rejection of the wine by the panelists and avoiding analytical problems (sensitivity). To overcome the effect of ethanol, which has been shown, might significantly affect the partition coefficient of the aroma compounds (Conner et al. 1998; Escalona et al. 1999; Rodriguez-Bencomo et al. 2002; Robinson et al. 2009; Villamor et al. 2013), all the wines, except the sweet wine were adjusted to the same ethanol concentration (13.5 % v/v). The differences in ethanol content between the sweet wine (15 % v/v) and the rest of the wines were of 1.5 % (v/v), which is unlikely to exert a significant difference on the release of the aroma compounds (Villamor et al. 2013). Wines were consumed following a systematic procedure in order to minimize inter-individual differences. Previous training sessions were performed with the panelists to familiarize them with the procedure. As it was described in the material and methods, a tailor-made breathing trapping device, previously optimized and validated for this purpose was employed. Although this type of device did not allow us to monitor the retronasal aroma release in real time such as the breath by breath analysis using mass spectrometric techniques (PTR-MS; APCI-MS), other important advantages such as the unequivocally identification of the compounds of interest, the possibility of having a concentrated breath extract (Buettner and Schieberle 2000) and the facility to adapt it to any laboratory with a relatively low economical investment make it very interesting for the purposes of this type of study.

The average data (relative peak areas) corresponding to each of the aroma released by the six panelists, independent of the type of wine, is depicted in **Figure 1**. Previously, the same consumption procedure was employed to evaluate the five wines without spiked aromas, and only traces of the four aroma compounds were detected. However, all the release data were corrected taking into consideration these results.

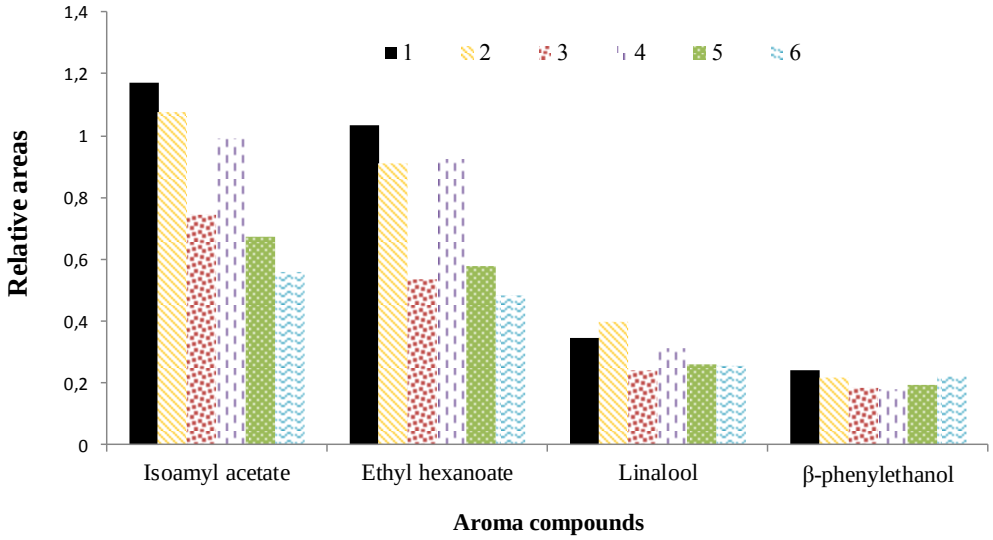


Figure 1. Average values of aroma release (relative peak areas) obtained for each panelist during wine consumption considering the five different wines (**WH-W**, **SP-W, YR-W, AR-W** and **SW-W**) and two repetitions of the same wine. For better comparison, data for *β*-phenylethanol are multiplied by a factor of 10. Different background patterns mean different panelists.

As can be observed in the figure, in spite that all the panelists followed a strict consumption procedure, for each single aroma compound, large differences in the released patterns between individuals were found. Interestingly, these differences seemed to be constant towards the same type of compound. For example, the inter-individual release pattern of the two esters, isoamyl acetate and ethyl hexanoate was quite similar; panelist #1, released the highest amount of these compounds, while panelist #6 always released the lowest. However, when considering a more chemically different compound, such as linalool, and *β*-phenylethanol, this pattern was different. For instance, linalool was more greatly released by panelists #1, #2, and #4, while the rest of subjects released almost the same amount. In addition, depending on the type of compound, differences between panelists were less pronounced, and for example, the release of *β*-phenylethanol was more or less the same for all of them. The existence of interindividual differences on aroma release patterns during food consumption have been extensively described, both in solid (Pionnier et al. 2004; Gierczynski et al. 2008; Blee et al. 2011) and liquid food-matrices (Buettner et al. 2001; Buettner et al. 2002; Deleris et al. 2011; Frank et al. 2011) and could be attributed to anatomical and physiological differences between panelists (respiratory flows, saliva composition, oral and pharyngeal mucosa, etc.).

To further investigate whether there was a trend in the aroma release pattern, a cluster analysis with the data corresponding to the aroma released by the 6 panelists during the consumption of the five different wines was performed. **Figure 2** shows the dendrogram obtained from this analysis in which two groups of aroma releasers can be clearly observed. A first group formed by panelists #1, #2 and #4, and a second comprising of panelists #3, #5 and #6. Both groups could be called higher and lower releasers respectively. A similar trend on flavor release was observed during the consumption of liquid emulsions (Frank et al. 2011) and these differences between higher and lower releasers were also in agreement with previous works.(Gierczynski et al. 2008; Blee et al. 2011) Although a higher number of individuals might be needed to confirm this trend, the preliminary results derived from this study indicated two behaviors on the aroma release during wine consumption.

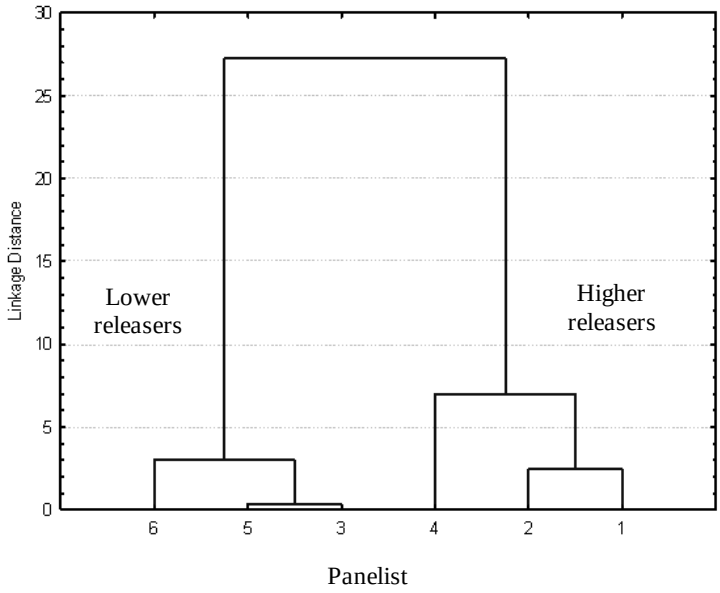


Figure 2. Dendrogram showing the grouping of panelists depending on their aroma release performance during the consumption of the five different types of wine.

Among the anatomical and physiological differences between panelists that might be responsible for the differences in the observed trends on aroma release, the variations in the flow rates between subjects might be an explanation. It has been suggested that a greater respiratory rate could contribute to bringing more volatiles to the upper airways, and consequently, more volatiles could be present in the expired air of the panelists (Hanaoka et al. 2001; Pionnier et al. 2004; Frank et al. 2011). However,

in another study performed *in vivo* and *in vitro*, Well et al (Weel et al. 2004) showed that an increase in flow rate resulted in a decrease in the aroma release.

To determine whether the breathing capacity might have an influence on the grouping of panelists in higher and lower releasers, two breathing related parameters corresponding to the vital capacity (VC) and forced vital capacity (FVC) were calculated. The vital capacity (VC) can be defined as the volume of air breathed out after the deepest inhalation (Langhammer et al. 2001), while the forced vital capacity (FVC) is the determination of the vital capacity from a maximally forced expiratory effort (Hedenström et al. 1985; Hedenström et al. 1986) In fact, FVC is the most basic maneuver in spirometry tests (Hedenström et al. 1985; Hedenström et al. 1986) Both parameters were estimated considering different individual physiological variables such as age, sex, ethnic group and height of the panelists. These data are shown in **Table 3.** As can be seen, the highest predicted FVC and VC values did indeed correspond to panelists from the first group (higher releasers), while the second group, the lower releasers, also showed the lowest FVC values. These results suggest that although other physiological variables might also affect the rate of aroma release during the consumption of wine, in the experimental conditions of this study, we have found a direct relationship between respiratory rate and aroma compounds in the expired air of the panelists, which might influence the sensory perception of wine aroma.

Table 3. Predicted Forced Vital Capacity (FVC) and Vital Capacity (VC) values calculated for the panelists[a].

[a] FVC and VC estimated using Dynamic Measurement Technologies software (www.dynamicmt.com) based on Hedenström et al., 1985-1986(Hedenström et al. 1985; Hedenström et al. 1986) and Langhammer et al.,2001,(Langhammer et al. 2001) respectively. SD: ± standard deviation

	FVC		VC	
Panelist	Predicted Value (L)	SD	Predicted Value (L)	SD
1	4.75	0.43	4.47	0.13
2	5.63	0.86	5.75	0.12
3	4.12	0.43	3.90	0.13
4	5.72	0.86	5.65	0.12
5	4.52	0.43	4.28	0.13
6	4.00	0.43	3.81	0.13

Effect of wine matrix composition on aroma release

Considering the two types of behaviors between panelists regarding the aroma release during wine consumption (higher and lower releasers), release data (relative peak areas) obtained from both groups of panelists were separately treated and submitted to one-way ANOVA in order to know if the non-volatile wine matrix composition could have a significant effect on aroma release during wine consumption. Surprisingly, we noticed that the effect of wine matrix was different depending on the group of panelists. For the higher releaser group, only the release of linalool was influenced by matrix composition (*0.05 < P < 0.1*). This result seemed to be related to some analytical constraints of the trapping device, such as the possible saturation of the Tenax trap during the experiment with higher releasers, which could have masked the wine matrix effect. However, considering the lower releaser group, all the compounds were significantly influenced by matrix composition; these results are shown in **Figure 3** together with the results corresponding to the application of the LSD test with the five types of wine matrices.

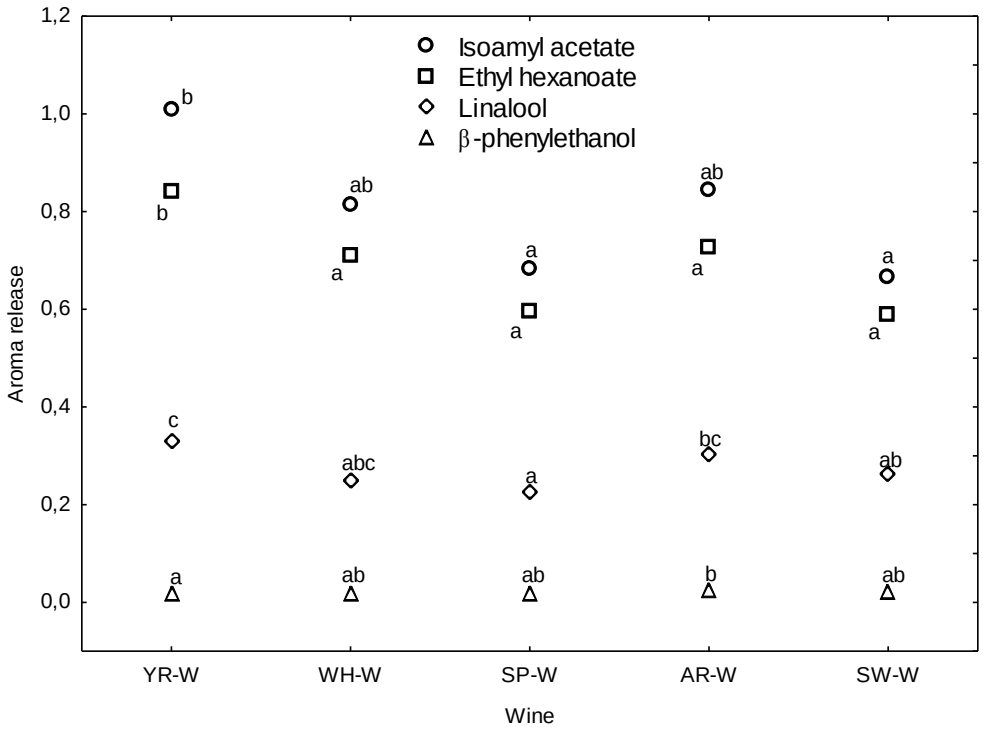


Figure 3. Results of the aroma release during the consumption of the five different types of wine for the lower releaser group. Significant differences on aroma release depending on matrix composition are indicated with * 0.05 < *P* < 0.1 or ** *P* < 0.05. Different letters across the different wines denotes statistical differences after the application of LSD test.

It is important to highlight that the differences on aroma release also depended on the type of aroma compound considered (e.g. we observed more differences on the

release of linalool towards the different wines that on the release of *β*-phenylethanol); however, independently on the type of aroma compound considered, there was a clear trend, in which higher aroma release was always observed during the consumption of the two red wines (**YR-W** and **AR-W**) compared to the consumption of white wines (**WH-W** and **SP-W**) and the sweet wine (**SW-W**). As can be seen in **Figure 3**, the highest release of isoamyl acetate, ethyl hexanoate, and linalool was during the consumption of young red wine, while *β*-phenylethanol was more released during the consumption of aged red wine.

Therefore, in spite of the inter-individual differences between panelists, the influence of wine matrix composition on the amount of aroma released during wine consumption was evidenced. This finding is in agreement with previous studies in the literature which have already demonstrated using static conditions, the existence of interaction between non-volatile wine matrix components and aroma compounds, which might affect the release of aroma compounds into the headspace (Dufour and Bayonove 1999; Dufour and Bayonove 1999; Dufour and Sauvaitre 2000; Aronson and Ebeler 2004; Rodriguez-Bencomo et al. 2011). Moreover, the effect of wine matrix on aroma release has been shown in *in vitro*- dynamic conditions (Genovese et al. 2009) being this the first time that this effect is proven in an *in vivo*, real consumption situation.

In order to try to determine which component/s from the wine matrix were more involved on aroma release during wine consumption, the chemical characterization of the non-volatile matrix composition of the five wines consumed by the panelists was performed and it is shown in **Table 4** together with the results of the LSD test. As can be seen, the 5 wines exhibited significant differences in their composition. As expected, the sweet wine (**SW-W**) was the most different, showing the highest pH (4.12), the lowest value of acidity (3.68 mg tartaric acid/L), and the highest content of neutral polysaccharides (171 g mannose/L) and residual sugars (310 g/L). In addition, this wine showed the highest levels of nitrogen compounds (total nitrogen, amino acids and peptides). In spite of its *a priori*, higher matrix complexity, during the consumption of this wine, we did not observe a significant effect on aroma release, which was more similar to that experienced during the consumption of white wines (**WH-W** and **SW-W**). The lack of a clear interaction effect of this type of wine with typical wine aroma

Table 4. Mean ± standard deviation (SD) values (*n = 3*) of the chemical composition of the five matrices: White wine (**WH-W)**, Sparkling wine (**SP-W),** Young red wine (**YR-W),** Aged red wine (**AR-W),** Sweet wine (**SW-W**)[a].

	WH-W		**SP-W**		**YR-W**		**AR-W**		**SW-W**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
pH	2.99[a]	0.0	3.07[b]	0.0	3.93[d]	0.0	3.66[c]	0.0	4.12[e]	0.0
Total acidity (mg tartaric acid/L)	5.60[e]	0.1	5.29[d]	0.1	4.06[b]	0.0	4.41[c]	0.1	3.68[a]	0.0
Total polyphenols (mg gallic acid/L)	211.50[a]	0.6	173.23[a]	5.2	2009.67[d]	52.0	1859.67[c]	20.9	574.33[b]	41.1
Neutral polysaccharides (g mannose/L)	1.82[a]	0.1	0.96[a]	0.1	2.66[a]	0.1	2.38[a]	0.1	171.07[b]	9.4
Residual sugars (g/L)	1.59[a]	0.0	1.14[a]	0.0	3.82[b]	0.3	2.58[ab]	0.3	310.08[c]	2.7
Total nitrogen (mg/L)	206.78[b]	3.8	129.36[a]	8.3	315.00[c]	51.1	358.68[c]	19.4	1010.80[d]	10.3
Amino acids + peptides (mg N/L)	64.51[a]	0.7	104.38[b]	0.9	141.05[d]	3.2	113.07[c]	2.5	319.61[e]	2.4
Amino acids (mg N/L)	36.39[a]	0.7	38.39[a]	0.4	69.59[c]	1.2	58.69[b]	3.2	67.20[c]	0.9
Peptides (mg N/L)*	28.12[a]		65.99[c]		71.46[d]		54.39[b]		252.41[e]	

[a,b,c,d] Different letters across the different wines denotes statistical differences after the application of LSD test. * This data is indirectly determined as the difference between the analytical determination of amino acids plus peptides and free amino acids, therefore SD values are not included in the table.

compounds is in agreement with previous results performed in static conditions (Rodriguez-Bencomo et al. 2011). It seems, at least, in static conditions, that the higher content of some small molecules in this wine, such as mono- and disaccharides or free amino acids (Delarue and Giampaoli 2006; Pozo Bayón and Reineccius 2009) could be responsible for a "salting out" effect which might compensate the retention effect exerted by other higher molecular weight compounds (such as polyphenols or proteins) (Pozo Bayón and Reineccius 2009; Rodriguez-Bencomo et al. 2011) or by an increase in the solution hydrophobicity because of the presence of a large amount of hydrophobic sugars such as fructose (Villamor et al. 2013).

The consumption of the two white wines (**WH-W, SP-W**) provided in general, a lower aroma release. These wines exhibited the highest values of acidity (5.6-5.29 mg tartaric/L) and therefore lowest pH values (2.99-3.07), although these differences did not seem enough to explain the differences observed on aroma release compared with the aroma release during red wines consumption. In addition, white and sparkling wines showed the lowest values of the majority of non-volatile compounds determined in the samples (polyphenols, polysaccharides, residual sugars, and nitrogen compounds). However, compared with red wines, the group of compounds that was dramatically different between the two types of samples was the content of total polyphenols. Red wines showed ten times more of these compounds (2010 and 1860 mg gallic acid /L for **YR-W** and **AR-W** respectively) compared to white wines (211 and 173 mg gallic acid/L for **WH-W** and **SP-W,** respectively).

To better envisage the compositional differences between the five types of wines, a principal component analysis (PCA) was performed with the data from **Table 4**. Two principal components were obtained. The first one (PC1), was negatively related (|loadings| > 0.82) with all the compositional parameters (pH, neutral polysaccharides, residual sugars, nitrogen compounds) except total polyphenols, and positively correlated with total acidity (0.92). The PC2 was positively correlated with the total polyphenol content (0.97). **Figure 4** shows the representation of the wines on the plane defined by PC1 and PC2. Clearly, PC1 could distinguish between sweet wines, with high and negative values for this component from white and sparkling wines with high and positive values for PC1. Red wines appeared together between sweet and white wines. On the other hand, PC2 clearly separated red wines from the rest. This component was

only defined by the polyphenol content, which clearly, as was previously commented, was much higher in these wines than in the rest.

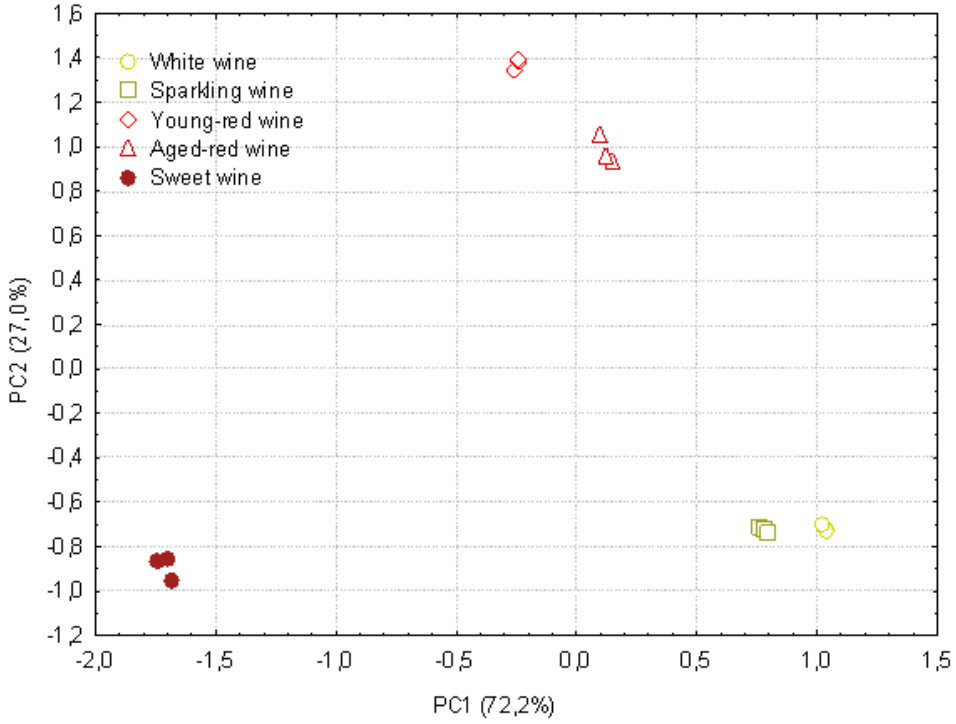


Figure 4. Representation of the wines on the plane defined by PC1 and PC2 obtained with the nonvolatile matrix composition data (Table 4).

Many works in the literature performed in static conditions have shown the existence of specific interactions between polyphenols and aroma compounds resulting in a reduction of the aroma released into the headspace (Dufour and Bayonove 1999; Aronson and Ebeler 2004; Villamor et al. 2013). For instance, it has been shown that monomeric polyphenols, abundant in young red wines, can interact with terpenes in ethanolic solutions provoking a lower aroma release in *in vitro* conditions (Dufour and Bayonove 1999). Other interactions that have been described involved the galloyl ring of some phenolic compounds and the aromatic ring of some aroma compound (Jung et al. 2000; Aronson and Ebeler 2004). Moreover, the type and polymerization state, which is related with the polyphenol origin (from seed or skin), might be involved in different interaction with aroma molecules (Mitropoulou et al., 2011; Lorrain et al., 2013). Anyway, in dynamic real drinking conditions, as we have used in this study, aroma release seems to be enhanced by the presence of polyhenols. One possible explanation could be that after swallowing, these types of compounds could specifically

interact with pharyngeal and/or oesophageal mucosa contributing to the formation of a product coating on the throat and pharynx, which could increase the contact area between air and product, favoring aroma release (Buettner et al. 2001) In fact, some works in the literature have already suggested interactions between aroma compounds and oral/ oesophageal mucosa to explain a delay on aroma release, which to an extent might be dependent on product composition (Buettner 2004; Deleris et al. 2011). Another explanation, could be the formation of polyphenol-aroma complexes in the surface coating, acting as aroma reservoirs and resulting in a greater concentration of aroma molecules ready to be released by the expiration flows. In fact, in *in vitro* systems (static headspace), using reconstituted wine samples, Mitropoulou and co-workers (Mitropoulou et al. 2011) suggested the possible formation of a protein-polyphenol-carbohydrate complex able to encapsulate hydrophobic aroma molecules.

Although obtaining straightforward relationships between the aroma release behavior during wine drinking and the effect of wine matrix components is a difficult task not only because of the differences in compositional parameters, but also because of the physicochemical characteristics of the aroma compounds and the physiological parameters affecting retronasal aroma release, a correlation analysis between compositional parameters (from **Table 4**) of all the wines of the study and the average values of aroma release considering the data from all the panelists was performed. Results showed that the matrix components which better correlated with the aroma release where the total polyphenol content, which were positively correlated with the release of isoamyl acetate (r = 0.81), ethyl hexanoate (r = 0.80) and linalool (r = 0.97). **Figure 5** shows this correlation, where it is possible to see how red wines showed the highest aroma release values, while the wines with the lowest polyphenol content showed the lowest aroma release for this compound. In spite of the greater aroma release that was observed by β-phenylethanol during the consumption of **AR-W**, the release of this compound did not correlate with the total polyphenol content or any other compositional parameter analyzed in the samples. This suggests that its release could be more affected by other chemicals of different nature or by specific interaction with different types of polyphenols more characteristic of aged wines (polymerized polyphenols) which will require further studies.

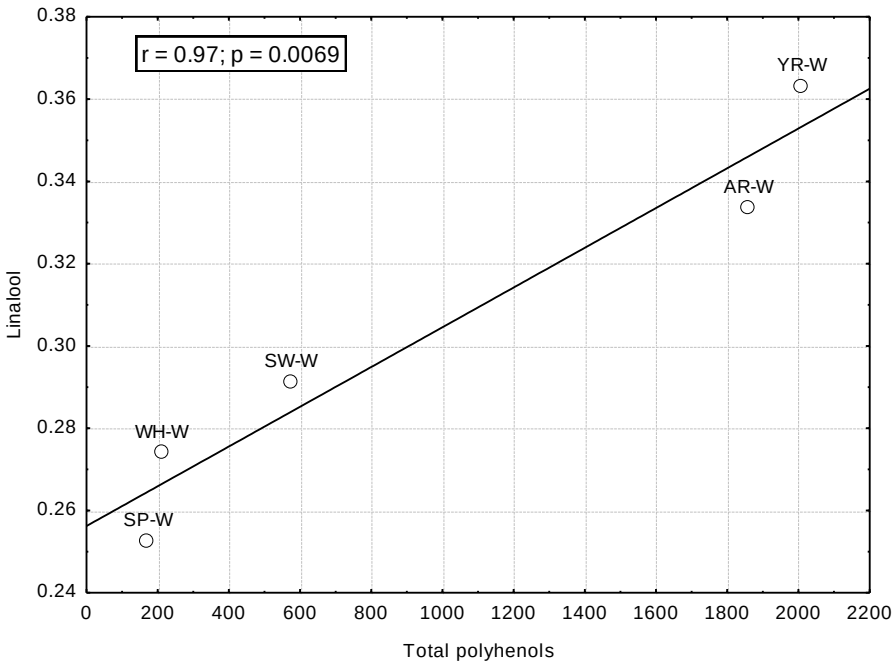


Figure 5. Correlation between the retronasal release of linalool during wine consumption and the polyphenol content in the five types of wines.

In conclusion, results of this work, show that in spite of the interindividual differences due to certain physiological characteristics of the individuals (such as the breathing capacity), there is an influence of the wine matrix composition on the *in vivo* aroma release from wines, and therefore in the amount of aroma available for the olfactory receptors. Among the different major wine matrix components, polyphenols, seem to enhance aroma release during wine drinking, which could be due to the involvement of these compounds on the formation of a wine coating after swallowing which might increase the contact area between exhaled air and product and/or because of the formation of polyphenol-aroma complexes on the surface coating acting as a reservoir of aroma molecules ready to be released by the expiration flows. Ongoing work is directed to study the nature of these interactions and their meaning for the sensory characteristics of the wines.

References

Aronson J, Ebeler SE (2004). Effect of Polyphenol compounds on the headspace volatility of flavors. American Journal of Enology and Viticulture 55(1): 13-21

Athes V, Lillo MPY, Bernard C, Perez-Correa R, Souchon I (2004). Comparison of experimental methods for measuring infinite dilution volatilities of aroma compounds in water/ethanol mixtures. J. Agric. Food Chem. 52(7): 2021-2027

Blee N, Linforth R, Yang N, Brown K, Taylor A (2011). Variation in aroma release between panellists consuming different types of confectionary. Flavour and Fragrance Journal 26(3): 186-191

Buettner A (2004). Investigation of potent odorants and afterodor development in two Chardonnay wines using the buccal odor screening system (BOSS). J. Agric. Food Chem. 52(8): 2339-2346

Buettner A, Beer A, Hannig C, Settles M (2001). Observation of the swallowing process by application of videofluoroscopy and real-time magnetic resonance imaging-consequences for retronasal aroma stimulation. Chemical Senses 26(9): 1211-1219

Buettner A, Beer A, Hannig C, Settles M, Schieberle P (2002). Physiological and analytical studies on flavor perception dynamics as induced by the eating and swallowing process. Food. Qual. Prefer. 13(7-8): 497-504

Buettner A, Schieberle P (2000). Exhaled odorant measurement (EXOM) - A new approach to quantify the degree of in-mouth release of food aroma compounds. Lebensm.-Wiss. Technol.-Food Sci. Technol. 33(8): 553-559

Conner JM, Birkmyre L, Paterson A, Piggott JR (1998). Headspace concentrations of ethyl esters at different alcoholic strengths. Journal of the Science of Food and Agriculture 77(1): 121-126

Delahunty CM, Piggott JR, Conner JM, Paterson A (1996). Comparison of dynamic flavour release from hard cheeses and analysis of headspace volatiles from the mouth with flavour perception during consumption. Journal of the Science of Food and Agriculture 71(3): 273-281

Delarue J, Giampaoli P (2006). Carbohydrate-flavour interactions. In: Voilley. A. and P. X. Etievant (eds). Flavour in Food. Boca Raton, Woohead, pp 208-224

Deleris I, Saint-Eve A, Guo Y, Lieben P, Cypriani M-L, Jacquet N, Brunerie P, Souchon I (2011). Impact of Swallowing on the Dynamics of Aroma Release and Perception during the Consumption of Alcoholic Beverages. Chemical Senses 36(8): 701-713

Dufour C, Bayonove C (1999). Interactions between Wine Polyphenols and Aroma Substances. An Insight at the Molecular Level. J. Agric. Food Chem. 47(2): 678-684

Dufour C, Bayonove CL (1999). Influence of wine structurally different polysaccharides on the volatility of aroma substances in a model system. J. Agric. Food Chem. 47(2): 671-677

Dufour C, Sauvaitre I (2000). Interactions between anthocyanins and aroma substances in a model system. Effect on the flavor of grape-derived beverages. J. Agric. Food Chem. 48(5): 1784-1788

Escalona H, Piggott JR, Conner JM, Paterson A (1999). Effect of ethanol strength on the volatility of higher alcohols and aldehydes. Italian Journal of Food Science 11(3): 241-248

Escudero A, Campo E, Farina L, Cacho J, Ferreira V (2007). Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. J. Agric. Food Chem. 55(11): 4501-4510

Frank D, Appelqvist I, Piyasiri U, Wooster TJ, Delahunty C (2011). Proton Transfer Reaction Mass Spectrometry and Time Intensity Perceptual Measurement of Flavor Release from Lipid Emulsions Using Trained Human Subjects. J. Agric. Food Chem. 59(9): 4891-4903

Genovese A, Piombino P, Gambuti A, Moio L (2009). Simulation of retronasal aroma of white and red wine in a model mouth system. Investigating the influence of saliva on volatile compound concentrations. Food Chem. 114(1): 100-107

Gierczynski I, Laboure H, Guichard E (2008). In vivo aroma release of milk gels of different hardnesses: Inter-individual differences and their consequences on aroma perception. J. Agric. Food Chem. 56(5): 1697-1703

Guth H (1997). Identification of character impact odorants of different white wine varieties. J. Agric. Food Chem. 45(8): 3022-3026

Hanaoka K, Vallet N, Giampaoli P, Heyd B, MacLeod P (2001). Possible influence of breathing on detection frequency and intensity rating in gas chromatography-olfactometry. Food Chem. 72(1): 97-103

Hedenström H, Malmberg P, H.V. F (1986). Reference Values for Lung Function tests in Men: Regression equations with Smoking Variables. Upsala J. Med. Sci. 91: 299-310
Hedenström H, Malmberg P, K. A (1985). Reference Values for Lung Function tests in Females. Bull. Eur. Physiopathol. Respir. 21: 551-557

Jones PR, Gawel R, Francis IL, Waters EJ (2008). The influence of interactions between major white wine components on the aroma, flavour and texture of model white wine. Food. Qual. Prefer. 19(6): 596-607

Jung DM, de Ropp JS, Ebeler SE (2000). Study of interactions between food phenolics and aromatic flavors using one- and two-dimensional H-1 NMR spectroscopy. J. Agric. Food Chem. 48(2): 407-412

Kotseridis Y, Baumes R (2000). Identification of impact odorants in Bordeaux red grape juice, in the commercial yeast used for its fermentation, and in the produced wine. J. Agric. Food Chem. 48(2): 400-406

Langhammer A, Johnsen R, Gulsvik A, Holmen TL, Bjermer L (2001). Forced spirometry reference values for Norwegian adults: the Bronchial Obstruction in North Trøndelag study. Eur. Respir. J. 18: 1-10

Lasekan O, Buettner A, Christlbauer M (2009). Investigation of the retronasal perception of palm wine (Elaeis guineensis) aroma by application of sensory analysis and exhaled odorant measurement (EOM). African Journal of Food, Agriculture, Nutrition and Development 9(2): 793-813

Lindinger W, Hansel A, Jordan A (1998). Proton-transfer-reaction mass spectrometry (PTR-MS): on-line monitoring of volatile organic compounds at pptv levels. Chem. Soc. Rev. 27(5): 347-354

Linforth R, Taylor AJ (2000). Persistence of volatile compounds in the breath after their consumption in aqueous solutions. J. Agric. Food Chem. 48(11): 5419-5423

Linforth RST, Savary I, Pattenden B, Taylor AJ (1994). Volatile compounds found in expired air during eating of fresh tomatoes and in the headspace above tomatoes. Journal of the Science of Food and Agriculture 65(2): 241-247

Lorrain B, Tempere S, Iturmendi N, Moine V, de Revel G, Teissedre PL (2013). Influence of phenolic compounds on the sensorial perception and volatility of red wine esters in model solution: An insight at the molecular level. Food Chem. 140(1-2): 76-82

Mitropoulou A, Hatzidimitriou E, Paraskevopoulou A (2011). Aroma release of a model wine solution as influenced by the presence of non-volatile components. Effect of commercial tannin extracts, polysaccharides and artificial saliva. Food Res. Int. 44(5): 1561-1570

Munoz-Gonzalez C, Rodriguez-Bencomo JJ, Moreno-Arribas MV, Pozo-Bayon MA (2011). Beyond the characterization of wine aroma compounds: looking for analytical approaches in trying to understand aroma perception during wine consumption. Anal. Bioanal. Chem. 401(5): 1497-1512

Pineau B, Barbe J-C, Van Leeuwen C, Dubourdieu D (2007). Which impact for beta-damascenone on red wines aroma? J. Agric. Food Chem. 55(10): 4103-4108

Pionnier E, Chabanet C, Mioche L, Le Quere JL, Salles C (2004). 1. In vivo aroma release during eating of a model cheese: Relationships with oral parameters. J. Agric. Food Chem. 52(3): 557-564

Polaskova P, Herszage J, Ebeler SE (2008). Wine flavor: chemistry in a glass. Chem. Soc. Rev. 37(11): 2478-2489

Pozo Bayón MA, Reineccius G (2009). Interactions Between Wine Matrix Macro-Components and Aroma Compounds In: M. V. Moreno-Arribas and M. C. Polo (eds). Wine Chemistry and Biochemistry Springer New York New York, pp 417-435

Robinson AL, Ebeler SE, Heymann H, Boss PK, Solomon PS, Trengove RD (2009). Interactions between Wine Volatile Compounds and Grape and Wine Matrix Components Influence Aroma Compound Headspace Partitioning. J. Agric. Food Chem. 57(21): 10313-10322

Rodriguez-Bencomo JJ, Conde JE, Rodriguez-Delgado MA, Garcia-Montelongo F, Perez-Trujillo JP (2002). Determination of esters in dry and sweet white wines by headspace solid-phase microextraction and gas chromatography. Journal of Chromatography A 963(1-2): 213-223

Rodriguez-Bencomo JJ, Munoz-Gonzalez C, Andujar-Ortiz I, Jose Martin-Alvarez P, Victoria Moreno-Arribas M, Angeles Pozo-Bayon M (2011). Assessment of the effect of the non-volatile wine matrix on the volatility of typical wine aroma compounds by headspace solid phase microextraction/gas chromatography analysis. Journal of the Science of Food and Agriculture 91(13): 2484-2494

Saenz-Navajas M-P, Campo E, Fernandez-Zurbano P, Valentin D, Ferreira V (2010). An assessment of the effects of wine volatiles on the perception of taste and astringency in wine. Food Chem. 121(4): 1139-1149

Saenz-Navajas MP, Campo E, Cullere L, Fernandez-Zurbano P, Valentin D, Ferreira V (2010). Effects of the Nonvolatile Matrix on the Aroma Perception of Wine. J. Agric. Food Chem. 58(9): 5574-5585

Taylor AJ, R.S. L (2000). Techniques for measuring volatile release in vivo during consumption of food. In: R. D.D. and T. A.J. (eds). Flavor Release. American Chemical Society, Washington D.C., pp 8-21

Villamor RR, Evans MA, Mattinson DS, Ross CF (2013). Effects of ethanol, tannin and fructose on the headspace concentration and potential sensory significance of odorants in a model wine. Food Res. Int. 50(1): 38-45

Weel KGC, Boelrijk AEM, Burger JJ, Verschueren M, Gruppen H, Voragen AGJ, Smit G (2004). New device to simulate swallowing and in vivo aroma release in the throat from liquid and semiliquid food systems. J. Agric. Food Chem. 52(21): 6564-6571

Publicación 4. La composición de la matriz vínica afecta la liberación temporal del aroma medida mediante PTR-ToF-MS.

Wine matrix composition affects temporal aroma release as measured by PTR-ToF-MS.

Carolina Muñoz-González, Etienne Semon, Gilles Feron, Elisabeth Guichard, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón.

Publicación enviada

Abstract

The aim of this study has been to evaluate the influence of wine non-volatile matrix composition on the temporal aroma release profile. To do so, a previously optimized procedure involving an artificial mouth coupled on line to a PTR-ToF-MS was used to monitor aroma release from five reconstituted desaromatized wines (white, sparkling, young-red, aged-red and sweet wines) with different non-volatile wine matrix composition but the same ethanol and aroma compounds content. The chemical wine matrix composition was also determined and results from the dynamic variables extracted from the aroma release curves (slope, I_{max}, AUC) of each aroma compound in each wine type were correlated with differences in wine matrix composition. Results showed a great influence of matrix composition on the temporal aroma release parameters. Red and sweet wines provided the highest and lowest aroma release, respectively. White non volatile matrix composition seemed to exert a minor effect on the temporal aroma release. Overall, these results confirmed that the physicochemical characteristics of the aroma compounds and wine matrix composition clearly seem to play an outstanding role in the temporal aroma release behavior.

Introduction

In a food product such as wine, aroma represents one of the most outstanding aspects related to quality and consumer preferences and choices. The non-volatile matrix of wine might exert a powerful influence on aroma release which may modify the amount of volatile compounds who reaches the olfactory receptors via orthonasal (odor) (Pineau et al. 2007) and retronasal (aroma release during wine consumption) (Munoz-Gonzalez et al. 2014). This "matrix effect" might have consequences on food aroma perception (Jones et al. 2008; Pozo-Bayón and Reineccius 2009; Sáenz-Navajas et al. 2010).

Most of the works performed in the effect of wine matrix composition on aroma release have been performed by using static headspace conditions evaluating the role of single wine matrix components (Escalona et al. 2001; Chalier et al. 2007; Mitropoulou et al. 2011) or using the whole wine matrix composition employing reconstituted wine samples (Rodriguez-Bencomo et al. 2011). The latter approach has the advantage of consider the great complexity of the wine matrix compared to the study of single wine matrix components. Although these works have provided valuable information related to the behavior of aroma compounds and the strength and nature of interactions between aroma compounds and some wine non-volatile components, they have not considered the dynamic nature of food consumption.

Likewise other liquid foods, wine consumption is a dynamic process, characterized by the presence of a continuous gas flow over time, and the influence of other physiological factors (saliva, changes in temperature, pH, and many other in-mouth oral parameters) that might change the aroma release profile of the food during the consumption and therefore, the perceived aroma. In this sense, although dynamic headspace conditions have been previously used to study interactions between some wine matrix components such as polyphenols and polysaccharides (Dufour and Bayonove 1999; Dufour and Bayonove 1999), the relatively recent work of Genovese et al, (Genovese et al. 2009) represents the first attempt to determine wine aroma release in closer *in mouth* conditions. Most recently, Muñoz-González et al. (2014b) have used a similar device to compare the effect of saliva on aroma release, taking into

consideration differences in wine matrix composition. In both studies it has been shown the outstanding role of saliva on wine aroma release.

In many of these studies the total aroma released after a fixed sampling time was taken as a measure of aroma release (cumulative way). However, aroma release is a sequential process in which the release can change in a short period of time. Therefore, the use of fast analytical techniques such as new mass spectrometric methods based on atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) and proton transfer reaction-mass spectrometry (PTR-MS), allow real-time analysis of volatile headspace concentration above a solution and therefore, they represent a valuable tool to study the temporal dimension of aroma release.

The characteristics of these techniques have been extensively described elsewhere (Taylor 1996; Lindinger et al. 1998), however, they have in common some advantages such as their good reproducibility, linearity, sensitivity, speediness, etc. While their use has been generalized in different types of food products, such as cheese (Pionnier et al. 2004; Feron et al. 2014), in the case of wines, the application of these techniques has been practically anecdotic (Muñoz-Gonzalez et al. 2011). One possible explanation may be due to some limitations of these techniques for wine aroma studies, due to the high and variable content of ethanol in this type of samples, which might produces the depletion of the reactant gas making difficult the comparison between samples with different ethanol content. However, different approaches in the methodology have been proposed to overcome this limitation (Aznar et al. 2004; Boscaini et al. 2004; Tsachaki et al. 2005; Fiches et al. 2014).

Real time mass spectrometry techniques can be used in *in vivo* or *in vitro* analysis. The use of human assessors for *in vivo* studies of retronasal aroma release, although very valuable has some important drawbacks, such as the interindividual differences or ethical considerations, among others. Therefore, the use of artificial devices to simulate *in mouth* or *in throat* conditions accounting for during liquid consumption is a very interesting methodological approach to overcome the problems associated to the use of human assessors and in combination with a fast MS technique, could be an interesting tool to study temporal aroma release from wine samples. Although *in vitro* mouth devices can never reflect the full complexity of what happens in mouth, they do combine a number of advantages for the study of release mechanisms

occurring in the oral cavity, such as the ability to control and modify variables, increased sensitivity (Roberts and Acree 1995; Deibler et al. 2001; Rabe et al. 2002) and no selectivity problems (Piggott and Schaschkeb 2001), among others.

PTR-MS has been mainly used in recent years to classify different kinds of food products based on their characteristic volatile mass fingerprint. Some of these studies include Mozzarella and Grana cheeses (Gasperi et al. 2001; Boscaini et al. 2004), juices (Biasioli et al. 2003), strawberries (Biasioli et al. 2003; Biasioli et al. 2003), olive oils (Araghipour et al. 2008) and butter oil (van Ruth et al. 2008). Only Lasekan et al, (Lasekan et al. 2009) have recently applied this methodology for the study of aroma release during the consumption of a type of fermented beverage obtained from Palm tree (called Palm wine). In addition Buettner et al. (Buettner et al. 2008) showed an application of this technique to study aroma release from two types of wines. Moreover, the coupling of a time-of-flight mass analyser to the PTR-MS system (PTR-ToF-MS) can improve the sensitivity and resolution of this technology enabling aroma release studies (Heenan et al. 2012) in complex food systems such as wine.

Taking these antecedents in mind, the objective of this work has been to study the influence of wine non-volatile matrix composition on the temporal aroma release profile using a previously optimized artificial mouth device coupled on-line to a PTR-ToF-MS. For this work, five different wines were lyophilized, desaromatized and reconstituted to the same ethanol content and spiked with a mixture of target aroma compounds. Wine matrix composition was also determined and results from the dynamic variables extracted from the aroma release curves (slope, I_{max}, AUC) of each wine type were discussed considering differences in wine matrix composition.

Material and methods

Aroma compounds

Twelve aroma compounds representative of the wine aroma profile were initially chosen for this investigation on the basis of their different physico-chemical characteristic more than on their aroma impact, since the objective of the work was to extract conclusions about the relationship between the type of aroma compound and the grade of interaction with the wine non volatile matrix. These compounds were 4 alcohols ((Z)-hex-3-en-1-ol, 1-butanol, isobutanol, 1-hexanol), 1 volatile phenol

(eugenol), 2 terpenes (α-terpineol, β-pinene), 1 lactone compound (furfural), 2 C13-norisoprenoids (β-damascenone, β-ionone) and 2 esters (ethyl dodecanoate and diethyl succinate). All the aroma compounds were of analytical grade (manufacturers: Aldrich, Fluka, Firmenich and Scharlau). Among them, ethyl dodecanoate was selected for this work as being representative of a very hydrophobic compound (high log *P* value) which could give us relevant information regarding its interaction with wine matrix. For each aroma compound, aroma stock solutions in ethanol absolute were prepared and from them each aroma compound was added at different concentration to the wine matrices. Previous studies were carried out in order to select the optimal concentration of the aroma compounds for the PTR-ToF-MS analysis. The selected concentrations of each of them allowed a good sensitivity avoiding instrument saturation (**Table 1**).

Wine samples

Five commercial Spanish wines representative of different winemaking technologies and therefore with different wine matrix compositions were selected for this study: a young Verdejo white wine (**WH-W**), a Cava white wine (Spanish sparkling wine manufactured by the traditional method) (**SP-W**), a young Tempranillo red wine (**YR-W**), a 4-year old (16 months in oak barrels) Tempranillo red wine (**AR-W**) and a sweet biologically aged wine made from Pedro Ximénez grapes (**SW-W**).

In addition to the commercial wines, a synthetic wine (**S-W**) representing a sample with 'no matrix effect' was prepared by mixing a hydroalcoholic solution with 4 g/L tartaric acid (Panreac, Barcelona, Spain) and adjusting the pH to 3.5 with NaOH (Panreac). This sample was further used for the optimization of the PTR-ToF-MS conditions.

Reconstituted wines

Deodorization procedure

Commercial wines were deodorized with Amberlite XAD-2 (Supelco, Bellefonte, PA, USA). Two 25 cm-length glass columns (Pobel, Madrid, Spain), one for each wine type, filled with 100 gr of Amberlite XAD-2 were prepared by sequentially conditioning with 250 mL of dichloromethane, then methanol and finally 375 mL of a 12 % (v:v) hydroethanolic solution. After this, wine samples were filtered through glass wool and loaded into the column by slowly passing 750 mL of each wine.

Deodorized wines (750 mL of each) were transferred to 250 mL vials and they were completely dried in a lyophiliser (Labconco, Kansas City, MO, USA). Five samples per wine type were prepared using this procedure. To replace the oxygen from the samples, all the dry samples were exposed to a Nitrogen atmosphere and stored at 4 ºC until sample preparation. This procedure practically ensured the complete elimination of the original aroma compounds in the wines as was confirmed by HS-SPME-GC/MS analysis.

Wine reconstitution

All the deodorized wines were reconstituted with the selected aroma compounds and with a hydroalcoholic solution to the same final ethanol concentration (12 % v/v). This procedure allowed having the same ethanol concentration in all the reconstituted wine samples, which results in two main advantages. By one hand, it equalized the proven effect of ethanol on aroma release which has the capacity to modify the solution polarity, thus altering the gas/liquid partition coefficient (Conner et al. 1998; Escalona et al. 1999; Rodriguez-Bencomo et al. 2002; Robinson et al. 2009). By the other hand, it avoided different reactivity and detection problems during the PTR-ToF-MS analysis due to differences in the amount of ethanol to react with the H_3O^+ reagent ions (Boscaini et al. 2004; Fiches et al. 2014).

Chemical wine matrix composition

Total acidity and pH, total polyphenols, neutral polysaccharides, residual sugar and nitrogen compounds (total nitrogen, free amino acids and peptides) were determined following previous analytical procedures (Rodriguez-Bencomo et al., 2011). **Table 2** shows the chemical composition of the wines employed in this study.

Table 1. Physicochemical characteristics and PTR-ToF-MS fragmentation patterns of the volatile compounds employed to aromatize the wine matrices.

Compounds	Physicochemical characteristics					Target mass			Concentration[f] (mg/L)	DESCRIPTOR[g]
	Chemical class[a]	Chemical formula	Mw[b]	log *P*[c]	BP[d] (ºC)		*Ion*	*m/z*[e]		
(Z)-hex-3-en-1-ol	1	$C_6H_{12}O$	100	1.61	156.5	$(M\text{-}H_2O)H^+$	$(C_6H_{10})H^+$	83.0855	1	grass
1-hexanol	1	$C_6H_{14}O$	102	2.03	156.0	$(M\text{-}H_2O)H^+$	$(C_6H_{12})H^+$	85.1012	0.1	resin, flower, green
β-ionone	2	$C_{13}H_{20}O$	192	3.84	262.9	MH^+	$C_{13}H_{21}O$	193.1587	1	raspberry, violet, flower
β-damascenone	2	$C_{13}H_{18}O$	190	4.21	275.0	MH^+	$C_{13}H_{19}O$	191.1430	4	apple, rose, honey
β-pinene	3	$C_{10}H_{16}$	136	4.35	164.0	MH^+	$C_{10}H_{17}$	137.1325	1	pine, resin, turpentine
Furfural	4	$C_5H_4O_2$	96	0.41	161.7	MH^+	$C_5H_5O_2$	97.0284	0.1	bread, almond, sweet
Ethyl dodecanoate	5	$C_{14}H_{28}O_2$	228	5.71	281.2	MH^+	$C_{14}H_{29}O_2$	229.2162	0.01	leaf
Eugenol	6	$C_{10}H_{12}O_2$	164	2.27	253.2	$(M\text{-}H_2O)H^+$	$(C_{10}H_{10}O)H^+$	147.0652	1	clove, honey

[a] Chemical class: 1-alcohol, 2-C13 norisoprenoid, 3-Terpene, 4-Aldehyde, 5-Ester and 6-Volatile phenol
[b] Molecular weight.
[c] Hydrophobic constant estimated using molecular modeling software EPI Suite (U.S. EPA 2000-2007).
[d] Boiling point estimated using molecular modeling software EPI Suite (U.S. EPA 2000-2007).
[e] Masses monitored by PTR-ToF-MS
[f] Final concentration assayed in the wines.
[g] From Flavornet (http://www.flavornet.org; accessed October 2009) database, from NIST web chemistry book (2005) (http://www.webbook.nis.gov/chemistry).

Human saliva

Stimulated human saliva was collected from 20 volunteers as described before (Poette et al. 2013; Poette et al. 2013). Participants should not consume food and water one hour before sampling. To stimulate production, volunteers chewed a little piece of Parafilm™ spitting out the saliva in a bottle as much as they could. Sodium azide (NaN_3, Sigma-Aldrich, Saint Quentin Fallavier, France) was added at a final concentration of 0.02% to avoid bacteria and fungi contamination and development. To obtain most representative salivary composition, the different saliva samples were pooled, mixed and centrifuged at 15000 g for 15 min. After that, the salivary pool was filtered through a 0.22 µm Sartorius device under vacuum at 4 ºC, to remove saliva bacteria. Finally, saliva was sampled in pots of 20 mL and stored at -80 ºC until use.

Dynamic in vitro aroma release

An artificial mouth device composed of a water-jacketed glass (100 mL) which allowed a temperature control of the sample set at 36 ºC was used to simulate the dynamic aroma release during wine consumption. As can be seen in **Figure 1** this device have five orifices: a) The first permits clean air to entry into the flask to purge the sample (100 mL min^{-1}), reproducing the dynamic conditions of the breathing phenomena, b) a second orifice is the purge gas outlet allowing the connection of the assembly to the inlet of the mass spectrometer, c) a third opening is where the sample is introduced, d) a fourth orifice allowed the introduction of a stir bar with digital speed control (150 rpm) in order to mix the sample as it might occur in the mouth and e) a fifth orifice allowed the empty of the system. Before being filled, reactor was cleaned three times with water and ethanol, dried and controlled for any residual volatile with PTR-ToF-MS.

Table 2. Chemical composition of the wines employed in this study**.**

	WH-W Mean (SD)	SP-W Mean (SD)	YR-W Mean (SD)	AR-W Mean (SD)	SW-W Mean (SD)
Non-volatile residue (g)	3.18 (0.17)	2.49 (0.07)	4.43 (0.11)	4.41 (0.09)	60.08 (0.82)
Non-volatile residue (% w/w)	2.17 (0.11)	1.72 (0.04)	2.99 (0.08)	2.96 (0.12)	36.3 (0.25)
pH	3.23 (0.02)	3.2 (0.00)	3.79 (0.04)	3.55 (0.03)	4.06 (0.01)
Total acidity (mg tartaric acid L^{-1})	5.66 (0.12)	4.67 (0.06)	4.29 (0.24)	4.84 (0.30)	3.99 (0.06)
Total polyphenols (mg gallic acid L^{-1})	269.95 (0.02)	175.83 (0.01)	1647.98 (0.29)	1672.62 (0.12)	765.36 (0.01)
Neutral polysaccharides (g mannose L^{-1})	1.67 (0.53)	0.80 (0.04)	2.50 (0.87)	2.27 (0.20)	219.32 (10.47)
Residual sugars (g L^{-1})	1.12 (0.23)	0.88 (0.11)	3.68 (0.52)	2.40 (0.23)	220.80 (6.91)
Total nitrogen (mg L^{-1})	239.96 (32.87)	254.94 (8.51)	406.00 (65.73)	382.76 (6.73)	798.84 (37.62)
Amino acids + peptides (mg N L^{-1})	49.54 (2.16)	96.33 (9.60)	133.51 (10.87)	100.17 (6.54)	309.37 (42.01)
Amino acids (mg N L^{-1})	30.67 (0.80)	41.48 (0.68)	58.57 (1.45)	56.13 (0.76)	77.59 (13.91)
Peptides (mg N L^{-1})*	18.87	54.85	74.94	44.04	231.78

Values are average of two determinations except for pH (average of three determinations).* This data is indirectly determined as the difference between the analytical determination of amino acids plus peptides and free amino acids, therefore SD values are not included in the table.

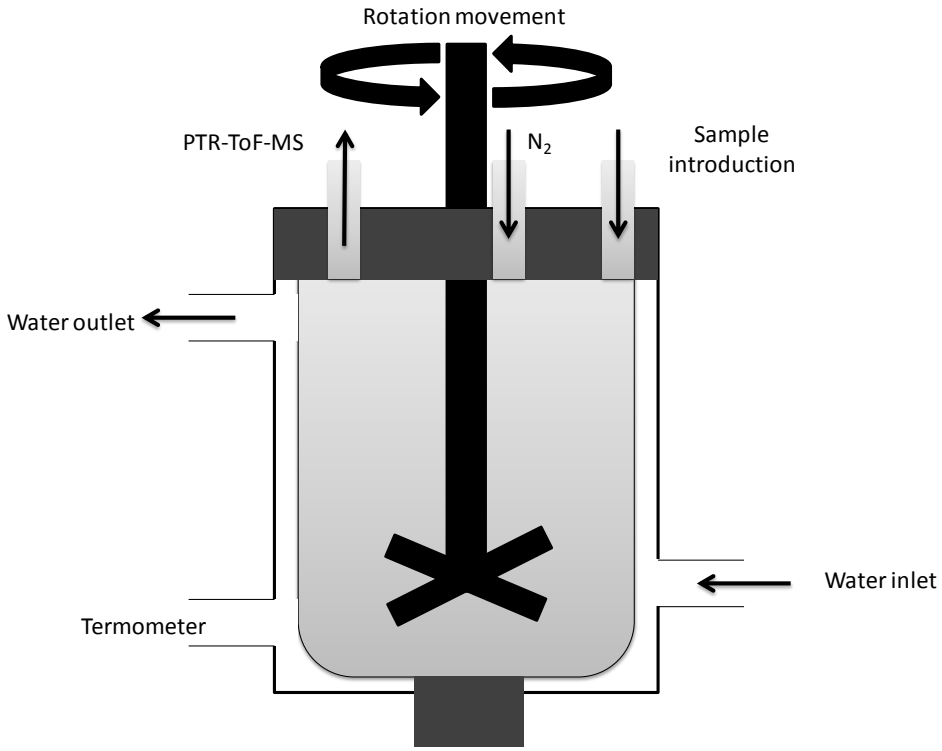


Figure 1. Schematic representation of the artificial mouth device used for coupling with a PTR-ToF-MS.

To better simulate oral conditions, human saliva was used in all the wines samples. This is an outstanding parameter that might differently affect aroma release depending on wine matrix composition (Genovese et al. 2009; Mitropoulou et al. 2011) Muñoz-Gonzalez et al., 2014b). The average ratio liquid food/saliva had previously shown to be 5/1 v/v (Genovese et al. 2009). Therefore, 10 mL of human saliva were transferred into the sample flask which was kept at 36 ºC. After that, 50 mL of the reconstituted wines (18 ºC) spiked with the volatile mixture were further added. Three replicates for each sample type were analyzed.

PTR-ToF-MS analysis

In vitro release kinetics was measured using a High-Resolution Proton Transfer Reaction-Time of Flight-Mass Spectrometer (PTR-ToF-MS) (Ionicon Analytik, Innsbruck, Austria). The PTR-ToF-MS instrument drift tube was thermally controlled (80 ºC) and operated with a voltage set at 480.1 (±0.3) V and a pressure of 2.42 mbar, resulting in a E/N ratio of 150.8 (±0.3) Td (E: electric field strength in the drift tube; N: buffer gas number density in the drift tube). Mass/charge ratios m/z 21 (signal for H_3O^+)

and m/z 37 (signal for water clusters H_2O^- H_3O^+) were monitored to check instrument performances and cluster ion formation.

Each headspace analysis lasted in total approximately 10 min, but for this study we only considered the first 30 s because this short sampling time might be more related to the initial introduction of the sample in the mouth (Rabe et al. 2004). For PTR-ToF-MS measurements, a constant air flow rate of 100 mL min^{-1} was used with 15 ml min^{-1} injected into the PTR-ToF-MS reaction chamber. Each measurement was preceded by the analysis of room air during 30 cycles.

For the determination of the fragmentation patterns of each of the studied aroma compounds, measurements were made in fullscan mode from mass/charge ratios m/z 0 to m/z 251.6, with a dwell time per mass of 1.08 s.

PTR-ToF-MS data acquisition and processing

For data acquisition, TOF DAQ software was applied. The time at which products were put in the artificial mouth was used as the reference for data comparison. The mean value from the last 30 measurement cycles was taken to account for potential background interferences. The parameters calculated were the maximal intensity of the released profile (I_{max30}), the slope ($Slope_{30}$) and the total area under curve (AUC_{30}) for the first 30 s of aroma release. As the objective was to compare the extent of aroma release between wines, the use of arbitrary units for aroma release data was sufficient for the analysis of intensity differences.

Statistical analysis

Aroma release data (absolute peak area) were submitted to one-way ANOVA to determine significant effects of the wine type. Differences between compounds were subsequently examined by least significant difference (LSD) test. Finally, a correlation analysis to determine the existence of correlations between each of the compositional variables and the aroma release data of the assayed aroma compounds. The significance level was $p < 0.05$ throughout the study. The STATISTICA program for Windows version 7.1 was used for data processing (StatSoft, Inc., 2005, www.statsoft.com).

Results and discussion

In previous studies it has been shown that the presence of saliva (Genovese et al. 2009; Mitropoulou et al. 2011; Muñoz-González, et al. 2014b) and wine matrix composition (Rodriguez-Bencomo et al. 2011; Muñoz-González et al. 2014b) might play an important role on aroma release in static and dynamic headspace conditions. Even, the latter effect has been demonstrated on *in vivo* conditions by the analysis of the exhaled breath of panelists after the consumption of aromatized wines (Muñoz-Gonzalez et al. 2014). However, none of these studies have taken into consideration the temporal characteristics of aroma release and the fact that aroma release might change during consumption due to differences in matrix composition. Therefore, in the present study, aroma release from five types of reconstituted wines, with the same amount of aroma compounds and ethanol, but different wine matrix composition was monitored by using an artificial mouth coupled with a PTR-ToF-MS.

Fragmentation patterns of volatile compounds in the PTR-ToF-MS

Although PTR-MS can be considered a soft ionization technique, fragmentation and cluster formation in the drift tube have been shown to happen (Deleris et al. 2011). Thus, the protonated molecular ion of an aroma compound might not be the most abundant mass. In addition, some volatile compounds might produce the same mass fragments, making more difficult a correct identification of the compounds. Therefore, preliminary experiments were conducted in order to select the most appropriate target mass from each aroma compound to further monitor it by PTR-ToF-MS. For these experiments a synthetic wine individually aromatized with the aroma compounds was used. Previous to the analysis, the headspace of the sample was equilibrated at room temperature for 30 minutes.

Several published works have reported changes in the ionization patterns of aroma compounds due to the presence of different content of ethanol in the samples (Boscaini et al. 2004; Aprea et al. 2007; Spitaler et al. 2007; Fiches et al. 2014). In this study, ethanol concentration was the same in all the studied wines; therefore, any change in the ionization conditions due to its presence should have the same effect in all the wine samples. In the present study, the fragmentation of ethanol leaded a serie of ethanol clusters with the same *m/z* than some of our target aroma compounds such as 1-butanol, isobutanol, α-terpineol and diethyl succinate, which could not be further

considered for this study. The fragmentation patterns for the rest of volatiles assayed are shown in **Table 1**. Here, the *m/z* target ions are also indicated. With this selection, no fragment overlapping was observed. Overall, the degree of fragmentation was low, with the protonated molecular ion being the most abundant mass for all the aroma compounds except for the linear alcohols. In the case of the alcohols ((Z)-hex-3-en-1-ol, 1-hexanol and eugenol) a major reaction pattern is the loss of a water molecule from the parent molecule, with rearrangement to yield a terminal alkene, but also clustering of the parent molecule with a hydronium ion might happen (**Table 1**). These results are in agreement with those published by Buhr and coworkers (Buhr et al. 2002) and Zardin et al. (Zardin et al. 2014). As a result, eight target aroma molecules still representing a wide range of physicochemical properties (**Table 1**) were finally selected for the following wine aroma release study using PTR-ToF-MS.

Influence of wine matrix composition on the temporal aroma release

An artificial mouth directly coupled to PTR-ToF-MS was used in this work to better simulate the main physiological factors influencing aroma release (liquid/gas phase ratio, presence of human saliva, air flows, temperature) during wine consumption, but avoiding the main drawbacks associated with the *in vivo* analysis (interindividual differences, ethical considerations, etc). **Figure 2** shows one example of the release profile of β-damascenone by using this *in vitro* approach in the five wine matrices considered in this study. It can be seen the dynamic parameters that can be extrapolated from the release curves (I_{max}, slope, AUC) and the differences in the release patterns observed among wines. To determine the effect of wine matrix composition on aroma release, a one-way ANOVA using the three dynamic parameters (AUC, I_{max} and slope) extracted from the aroma release curves of each wine type was performed. These results and the average values calculated for the three parameters in the five types of wine samples are shown in **Table 3**. As it can be seen, all the aroma compounds were significantly ($p < 0.05$) influenced by wine matrix composition except the two lineal alcohols (Z)-hex-3-en-1-ol and 1-hexanol. These are polar compounds with relatively low log *P* values, and characterized by low molecular weights and boiling points. The little effect of wine matrix composition observed for the alcohols is in agreement with results from previous works performed in static (Rodriguez-Bencomo et al. 2011), and dynamic conditions (Muñoz-González et al. 2014b). However, it is important to

highlight that this result might be also related to the high variability of the PTR-MS measurement due to the major fragmentation of these compounds.

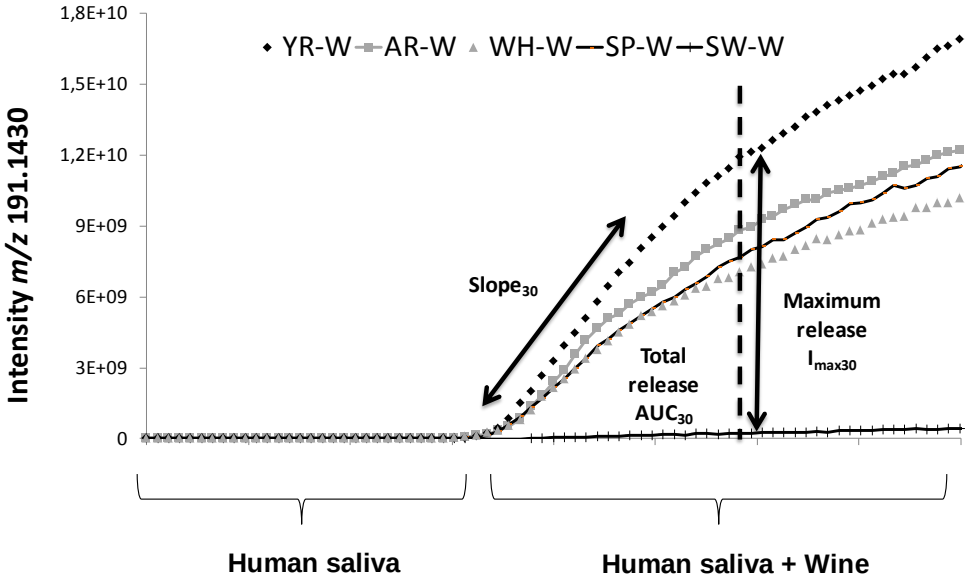


Figure 2. Example of β-damascenone release from wines with different composition by using the artificial mouth coupled to PTR-ToF-MS (YR-W: young-red wine; AR-W: aged-red wine; WH-W: white wine; SP-W: sparkling wine; SW-W: sweet wine).

Table 3 also shows the results for the mean comparison (LSD test) of each aroma compound in the different type of wines considering the three aroma release parameters. In general, the three of them showed similar results, indicating a high correlation between them that has been already noticed in others works (Pozo-Bayón et al. 2009). As it can be seen, the release behavior of the aroma compounds in the different wine matrices was largely different. As expected, sweet wines showed the lowest values for I_{max}, AUC and slope for all the aroma compounds (except for eugenol) being the wine matrix with the highest retention effect. This could be related to its complex matrix composition characterized by the highest non-volatile residue (36.3 % w/w), which could be due to the high content of sugars and nitrogen compounds (total nitrogen and amino acids) (**Table 2**).

Table 3. Average values corresponding to the dynamic release parameters (Imax, Slope, AUC) obtained by PTR-ToF-MS in the five types of wine matrices.

Compounds		White wine	Sparkling wine	Young-Red Wine	Aged-Red Wine	Sweet wine
(Z)-Hex-3-en-1-ol	$Slope_{30}$	0.94	0.91	2.18	1.40	0.21
	AUC_{30}	440.00	414.88	1028.90	661.00	96.42
	$Imax_{30}$	28.43	27.71	67.14	43.55	6.70
1-hexanol	$Slope_{30}$	0.66	0.72	2.02	1.20	0.23
	AUC_{30}	324.27	375.75	1329.36	638.84	114.36
	$Imax_{30}$	20.50	23.48	65.84	37.34	7.06
β-ionone	$Slope_{30}$	**4.19 b**	**4.79 b**	**7.95 c**	**4.90 b**	**1.12 a**
	AUC_{30}	**1935.55 b**	**2161.03 b**	**3537.83 c**	**2006.49 b**	**498.48 a**
	$Imax_{30}$	**128.94 b**	**144.79 b**	**240.35 c**	**148.22 b**	**34.17 a**
β-damascenone	$Slope_{30}$	**25.53 b**	**28.47 b**	**43.64 c**	**31.12 b**	**10.23 a**
	AUC_{30}	**12428.01 b**	**13458.55 b**	**21032.37 c**	**14077.72 b**	**4553.49 a**
	$Imax_{30}$	**774.36 b**	**861.08 b**	**1319.67 c**	**942.75 b**	**311.95 a**
β-pinene	$Slope_{30}$	**6.69 a**	**9.72 b**	**23.09 d**	**17.98 c**	**4.94 a**
	AUC_{30}	**4115.57 ab**	**6164.09 b**	**15140.68 d**	**10182.06 c**	**2580.55 a**
	$Imax_{30}$	**204.78 ab**	**297.78 b**	**754.03 d**	**547.21 c**	**147.75 a**
Furfural	$Slope_{30}$	**1594.15 ab**	**1858.62 bc**	**2457.73 c**	**2050.14 bc**	**923.68 a**
	AUC_{30}	**93.94 ab**	**124.27 bc**	**177.06 c**	**135.30 bc**	**61.90 a**
	$Imax_{30}$	**3.08 ab**	**4.11 bc**	**5.86 c**	**4.47 bc**	**2.05 a**
Ethyl dodecanoate	$Slope_{30}$	**32.14 c**	**26.81 ab**	**25.13 b**	**21.38 b**	**3.31 a**
	AUC_{30}	**13701.63 c**	**12166.16 bc**	**10129.70 ab**	**8610.99 b**	**1584.80 a**
	$Imax_{30}$	**971.84 c**	**810.77 bc**	**760.00 b**	**646.64 b**	**100.47 a**
Eugenol	$Slope_{30}$	0.08	0.47	0.25	0.36	0.10
	AUC_{30}	**39.49 a**	**286.63 b**	**134.32 ab**	**160.12 ab**	**61.74 a**
	$Imax_{30}$	3.23	16.99	9.20	11.14	4.07

All values are divided by a factor of 10^7. Values statistically significantly different ($p < 0.05$) among different wine matrices are indicated in bold. Different letters for the same aroma compound denote statistical differences among wine matrices after applying LSD test.

The use of dry grapes and the biological aging process necessary for the production of sweet wines can be responsible for the high content of these compounds. In addition, these wines, which are submitted to an aging process in presence of lees, might contain a relatively elevated content of yeast manoproteins (Charpentier and Feuillat 1993; (Martinez-Rodriguez and Polo 2000), with aroma binding capacity (Langourieux and Crouzet 1997; Chalier et al. 2007). The intensity of this effect might depend on the nature of the manoprotein and on the physico-chemical properties of the aroma compounds (Charlier et al. 2007). In fact, it has been suggested that the more hydrophobic compounds seem to show greater binding capacities, suggesting that the degree of binding is of hydrophobic nature (Lubbers et al. 1994). This observation is in agreement with our results which showed the greater retention (lower values of I_{max}, AUC, slope) for ethyl dodecanoate which was the most hydrophobic compound. Moreover, sweet wines with saliva showed the highest values of viscosity as shown in **Table 4** (due to its complex matrix composition) which could be also related with a minor aroma release as previously shown by (Roberts and Acree 1996).

Table 4. Viscosity and pH values determined for the wine/saliva mixtures (n=3).

	Viscosity(mPa x s) Mean (SD)	**pH** Mean (SD)
White wine	7.85 (0.1)	3.3 (0.1)
Sparkling wine	7.83 (0.2)	3.4 (0.1)
Young-red Wine	7.97 (0.2)	4.0 (0.1)
Aged-red Wine	7.83 (0.1)	3.8 (0.1)
Sweet wine	10.07 (0.2)	4.1 (0.1)

Viscosity values measured at room temperature, 1500 s^{-1}, 40 s (LAMY RHEOLOGY RHÉOMAT RM 200).

Contrarily, as it can be seen in **Table 3**, young-red wines showed the highest dynamic release parameters for most of the aroma compounds assayed (β-ionone; β-damascenone; β-pinene, furfural). The higher aroma release from red wines compared to white and sweet wines has been recently shown using a retronasal trapping aroma device in *in vivo* conditions (Muñoz-González et al. 2014). This effect could be attributed to the high total polyphenol content (1647.98 mg L^{-1}) in this wine compared to the others (269.95, 175.83, 765.36 mg L^{-1}) for white, sparkling, and sweet wines respectively) (**Table 2**). In this regard, different works in the literature have shown the existence of specific interactions between polyphenols and aroma compounds resulting in both, a reduction in the aroma release or an enhancement (salting-out effect)

depending on the aroma compound and the concentration of polyphenols assayed (Dufour and Bayonove 1999; Aronson and Ebeler 2004; Mitropoulou et al. 2011; Villamor et al. 2013). However, most of these works have been performed in static conditions and, even more important, they have been performed considering only some matrix components, which although is a very valuable approach, it did not consider the dynamic conditions of food consumption and the large complexity of the wine matrix. In this study, using a dynamic approach and considering the whole wine matrix composition, aroma release seems to be enhanced by the presence of polyphenols, which is in agreement with the previously mentioned *in vivo* study. This effect could be due to the presence of polyphenols and polysaccharides and the formation of large complexes (saliva protein–wine polyphenol–wine carbohydrate) able to encapsulate hydrophobic aroma compounds (Mitropoulou et al. 2011). These complexes might retained more aroma molecules in red wines, ready to be released in dynamic conditions (*in vitro* or *in vivo*). However, in spite of the similar polyphenol content (1672.62 mg L^{-1}), aroma release in aged-red wines was not as higher as for young-red wines. This could be due to differences in the type and polymerization state of wine polyphenols which might influence the interaction mechanisms with the aroma compounds (Mitropoulou et al. 2011;(Lorrain et al. 2013)). Nonetheless, both red wines showed the highest dynamic aroma release parameters compared to the other three types of wines. Besides the possible binding of some aroma compounds into protein-polyphenol-carbohydrate macrocomplexes, the higher aroma release observed in the young-red wine could be the result of aggregation and/or precipitation of tannins (typically, astringent compounds) with salivary proteins, such as mucins (Mitropoulou et al. 2011) or PRPs (proline rich proteins) (Canon et al. 2013), which might result in a salting-out effect (**Table 3**). However other compounds such as ethyl dodecanoate or eugenol did not seem to follow this trend, possibly due to other ways of interaction with the matrix components.

White wines (white and sparkling wine) showed in general, an intermediate aroma release behavior between sweet and red wines. The lower amount of polyphenols and carbohydrates (**Table** 2) could not favor as much the formation of the above cited aroma encapsulation complexes. The lower aroma release in white wines compared to red wines is in agreement with previous work performed in *in vivo* conditions (Muñoz-González et al. 2014). Viscosity values for red and white wines with saliva were quite

similar (**Table 4**), so this did not seem a key factor that could affect aroma release in these wines.

In order to better visualize the impact of wine matrix composition on the temporal aroma release, and taking into consideration the correlation among the three dynamic release parameters extracted from the release curves, **Figure 3** shows the comparison of the AUC values for the compounds significantly affected by the wine matrix composition in each wine type. As it can be seen, the release behavior of the compounds seemed to be constant toward the same chemical class and wine type. For example, the AUC values followed a similar pattern for the two C13-norisoprenoids (β-damascenone and β-ionone), showing the highest AUC values in the young-red wine, followed by the aged-red, sparkling and white wine. The lowest AUC values were observed for the sweet wine. Specifically, in the case of β-damascenone, above 78 % of reduction in AUC values among young-red and the sweet wine was found, which underline the large impact of wine matrix composition for this chemical family of compounds. Considering other aroma chemical classes, such as terpenes (β-pinene) or aldehydes (furfural), young-red wine was again the wine that showed the greatest AUC values, while the sweet wine showed the lowest. However, the differences among red and white wines in the release of furfural were less evident than for β-pinene. The latter might be more affected by wine matrix composition, at least in the real time dynamic conditions employed in this study.

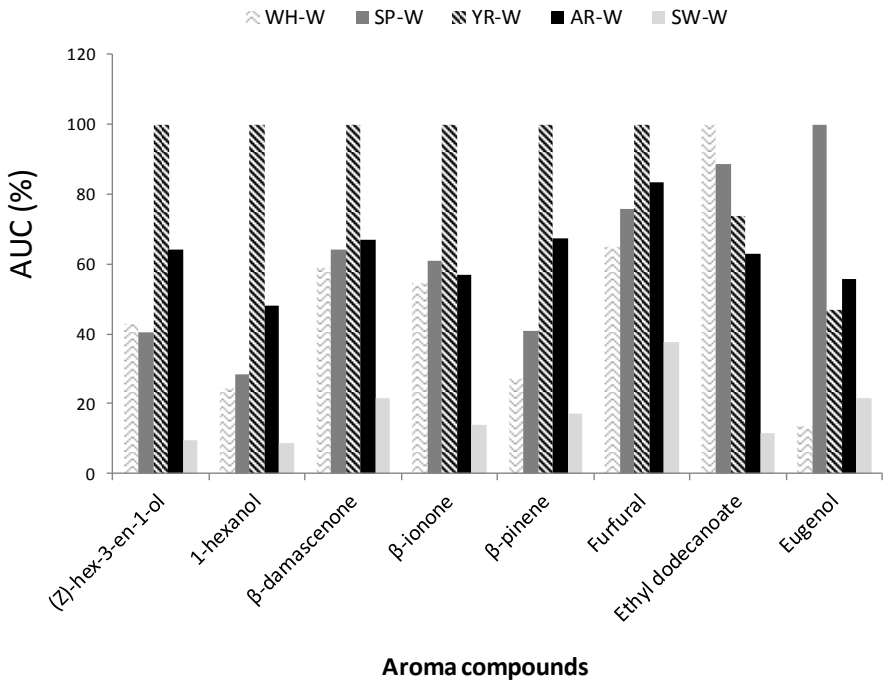


Figure 3. Difference in AUC values (%) for the aroma compounds significantly affected by wine matrix composition (YR-W: young-red wine; AR-W: aged-red wine; WH-W: white wine; SP-W: sparkling wine; SW-W: sweet wine).

However, when considering the behavior of ethyl dodecanoate, the most hydrophobic compound assayed in this study, the highest aroma release was determined in the white wine, while it was higher retained in red, sweet and sparkling wines (**Figure 3**). This effect might be related to differences in matrix composition. For example, it has been previously shown that tannins at higher concentration (10 g L^{-1}) could form colloidal size particles decreasing the volatility of ethyl dodecanoate in the headspace (Mitropoulou et al., 2011). However, the same authors proven that at lower concentrations (0-5 g L^{-1}) a marked salting-out effect, specifically for ethyl dodecanoate, was evidenced. Moreover, the lower amount of nitrogen compounds in the white wine (**Table 2**) might favor the lower retention and therefore the highest release of this hydrophobic compound.

Finally, the volatile phenol, eugenol, also showed a significant effect among the different wine matrices (**Figure 3**). Interestingly, eugenol was the only compound more released in the sparkling wine than in the other matrices. This is an aromatic compound that can establish π-π interactions with wine polyphenols (Jung and Ebeler 2003), resulting in a decrease of this compound in the headspace of the solution. The

reconstituted sparkling wine showed the lowest values of polyphenols (**Table 2**) which could explain this result and its lower release from red wine matrices.

For a deeper understanding on the relationship between wine non-volatile matrix composition and the parameters more influenced in the dynamic aroma release, a correlation study was performed with the compositional parameters of all the wines and the average aroma release values considering the three dynamic release parameters (I_{max}, AUC and slope) (data not shown). This study showed that the only aroma compound correlated with the matrix component was ethyl dodecanoate which was negatively and highly correlated |> 0.91| with neutral polysaccharides, residual sugars and all the nitrogen compounds. These results seem to confirm the hypothesis that a higher matrix complexity, such in the case of sweet wines, could be related to a retention effect on aroma release, and mainly observed for very hydrophobic compounds. Conversely, in the white wine the low level of these compounds could be the responsible for the highest release of ethyl dodecanoate.

Conclusions

The experimental methodology employed in this study (using reconstituted wines keeping the whole original wine matrix composition but adjusting the level of aroma and ethanol) allowed the real time aroma release monitoring by using PTR-MS-ToF, which better simulate the real situation produced during the *in vivo* consumption. By using this technique it has been shown the great influence of wine non volatile matrix composition on the dynamic of aroma release. The highest retention (lower release) of aroma was found in the wines with the highest contents of nitrogen compounds and sugars (sweet wines), while the two red wines with the highest total polyphenol content showed the highest aroma release. Overall, the results of this study might contribute to explain the effect of wine non volatile composition in the first moments of in-mouth wine processing which could be tightly related to wine aroma perception. Oncoming work will be devoted to determine the meaning of this effect at sensory level.

References

Aprea E, Biasioli F, Mark TD, Gasperi F (2007). PTR-MS study of esters in water and water/ethanol solutions: fragmentation patterns and partition coefficients. International Journal of Mass Spectrometry 262(1-2): 114-121

Araghipour N, Colineau J, Koot A, Akkermans W, Rojas JMM, Beauchamp J, Wisthaler A, Maerk TD, Downey G, Guillou C, Mannina L, Van Ruth S (2008). Geographical origin classification of olive oils by PTR-MS. Food Chemistry 108(1): 374-383

Aronson J, Ebeler SE (2004). Effect of polyphenol compounds on the headspace volatility of flavors. American Journal of Enology and Viticulture 55(1): 13-21

Aznar M, Tsachaki M, Linforth R, Ferreira V, Taylor A (2004). Headspace analysis of volatile organic compounds from ethanolic systems by direct APCI-MS. International Journal of Mass Spectrometry 239(1): 17-25

Biasioli F, Gasperi F, Aprea E, Colato L, Boscaini E, Mark TD (2003). Fingerprinting mass spectrometry by PTR-MS: heat treatment vs. pressure treatment of red orange juice - a case study. International Journal of Mass Spectrometry 223(1-3): 343-353

Biasioli F, Gasperi F, Aprea E, Mott D, Boscaini E, Mayr D, Mark TD (2003). Coupling proton transfer reaction-mass spectrometry with linear discriminant analysis: a case study. Journal of Agricultural and Food Chemistry 51(25): 7227-7233

Boscaini E, Mikoviny T, Wisthaler A, von Hartungen E, Mark TD (2004). Characterization of wine with PTR-MS. International Journal of Mass Spectrometry 239(2-3): 215-219

Buettner A, Otto S, Beerc A, Mestres M, Schieberle P, Hummel T (2008). Dynamics of retronasal aroma perception during consumption: Cross-linking on-line breath analysis with medico-analytical tools to elucidate a complex process. Food Chemistry 108: 1234-1246

Buhr K, van Ruth S, Delahunty C (2002). Analysis of volatile flavour compounds by Proton Transfer Reaction-Mass Spectrometry: fragmentation patterns and discrimination between isobaric and isomeric compounds. International Journal of Mass Spectrometry 221(1): 1-7

Canon F, Pate F, Cheynier V, Sarni-Manchado P, Giuliani A, Perez J, Durand D, Li J, Cabane B (2013). Aggregation of the Salivary Proline-Rich Protein IB5 in the Presence of the Tannin EgCG. Langmuir 29(6): 1926-1937

Conner JM, Birkmyre L, Paterson A, Piggott JR (1998). Headspace concentrations of ethyl esters at different alcoholic strengths. Journal of the Science of Food and Agriculture 77(1): 121-126

Chalier P, Angot B, Delteil D, Doco T, Gunata Z (2007). Interactions between aroma compounds and whole mannoprotein isolated from Saccharomyces cerevisiae strains. Food Chemistry 100(1): 22-30

Deibler K, Lavin E, Linforth R, Taylor AJ, Acree TE (2001). Verification of a mouth simulator by in vivo measurements. J. Agric. Food Chem. 49(3): 1388–1393

Deleris I, Saint-Eve A, Guo Y, Lieben P, Cypriani M-L, Jacquet N, Brunerie P, Souchon I (2011). Impact of Swallowing on the Dynamics of Aroma Release and Perception during the Consumption of Alcoholic Beverages. Chemical Senses 36(8): 701-713

Dufour C, Bayonove C (1999). Interactions between Wine Polyphenols and Aroma Substances. An Insight at the Molecular Level. Journal of Agricultural and Food Chemistry 47(2): 678-684

Dufour C, Bayonove CL (1999). Influence of wine structurally different polysaccharides on the volatility of aroma substances in a model system. Journal of Agricultural and Food Chemistry 47(2): 671-677

Escalona H, Homman-Ludiye M, Piggott JR, Paterson A (2001). Effect of potassium bitartrate, (+)-catechin and wood extracts on the volatility of ethyl hexanaote and octanal in ethanol/water solutions. Lebensmittel-Wissenschaft und-Technologie 34(2): 76-80

Escalona H, Piggott JR, Conner JM, Paterson A (1999). Effect of ethanol strength on the volatility of higher alcohols and aldehydes. Italian Journal of Food Science 11(3): 241-248

Feron G, Ayed C, Qannari EM, Courcoux P, Laboure H, Guichard E (2014). Understanding Aroma Release from Model Cheeses by a Statistical Multiblock Approach on Oral Processing. Plos One 9(4)

Fiches G, Deleris I, Saint-Eve A, Brunerie P, Souchon I (2014). Modifying PTR-MS operating conditions for quantitative headspace analysis of hydro-alcoholic beverages. 2. Brandy characterization and discrimination by PTR-MS. International Journal of Mass Spectrometry 360(1): 15-23

Gasperi F, Gallerani G, Boschetti A, Biasioli F, Monetti A, Boscaini E, Jordan A, Lindinger W, Iannotta S (2001). The mozzarella cheese flavour profile: a comparison between judge panel analysis and proton transfer reaction mass spectrometry. Journal of the Science of Food and Agriculture 81(3): 357-363

Genovese A, Piombino P, Gambuti A, Moio L (2009). Simulation of retronasal aroma of white and red wine in a model mouth system. Investigating the influence of saliva on volatile compound concentrations. Food Chemistry 114(1): 100-107

Heenan S, Soukoulis C, Silcock P, Fabris A, Aprea E, Cappellin L, Maerk TD, Gasperi F, Biasioli F (2012). PTR-TOF-MS monitoring of in vitro and in vivo flavour release in cereal bars with varying sugar composition. Food Chemistry 131(2): 477-484

Jones PR, Gawel R, Francis IL, Waters EJ (2008). The influence of interactions between major white wine components on the aroma, flavour and texture of model white wine. Food Quality and Preference 19(6): 596-607

Jung DM, Ebeler SE (2003). Headspace solid-phase microextraction method for the study of the volatility of selected flavor compounds. Journal of Agricultural and Food Chemistry 51(1): 200-205

Langourieux S, Crouzet J (1997). Study of interactions between aroma compounds and glycopeptides by a model system. J. Agric. Food Chem 45(5): 1873-1877

Lasekan O, Buettner A, Christlbauer M (2009). Investigation of the retronasal perception of palm wine (Elaeis guineensis) aroma by application of sensory analysis and exhaled odorant measurement (EOM). African Journal of Food, Agriculture, Nutrition and Development 9(2): 793-813

Lindinger W, Hansel A, Jordan A (1998). On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS) medical applications, food control and environmental research. International Journal of Mass Spectrometry and Ion Processes 173(3): 191-241

Lorrain B, Tempere S, Iturmendi N, Moine V, de Revel G, Teissedre P-L (2013). Influence of phenolic compounds on the sensorial perception and volatility of red wine esters in model solution: An insight at the molecular level. Food Chemistry 140(1-2): 76-82

Lubbers S, Charpentier C, Feuillat M, Voilley A (1994). Influence of yeast walls on the behavior of aroma compounds in a model wine. American Journal of Enology and Viticulture 45(1): 29

Martinez-Rodriguez AJ, Polo MC (2000). Characterization of the nitrogen compounds released during yeast autolysis in a model wine system. Journal of Agricultural and Food Chemistry 48(4): 1081-1085

Mitropoulou A, Hatzidimitriou E, Paraskevopoulou A (2011). Aroma release of a model wine solution as influenced by the presence of non-volatile components. Effect of commercial tannin extracts, polysaccharides and artificial saliva. Food Research International 44(5): 1561-1570

Munoz-Gonzalez C, Martin-Alvarez PJ, Victoria Moreno-Arribas M, Angeles Pozo-Bayon M (2014). Impact of the Nonvolatile Wine Matrix Composition on the In Vivo Aroma Release from Wines. Journal of Agricultural and Food Chemistry 62(1): 66-73

Muñoz-Gonzalez C, Rodriguez-Bencomo JJ, Moreno-Arribas MV, Pozo-Bayon MA (2011). Beyond the characterization of wine aroma compounds: looking for analytical approaches in trying to understand aroma perception during wine consumption. Analytical and Bioanalytical Chemistry 401(5): 1497-1512

Piggott J, Schaschkeb C (2001). Release cells, breath analysis and in-mouth analysis in flavour research. Biomolecular Engineering 17(4-5): 129-136

Pineau B, Barbe JC, Van Leeuwen C, Dubourdieu D (2007). Which impact for b-damascenone on red wines aroma? Journal of Agricultural and Food Chemistry 55(10): 4103-4108

Pionnier E, Chabanet C, Mioche L, Le Quere JL, Salles C (2004). 1. In vivo aroma release during eating of a model cheese: Relationships with oral parameters. Journal of Agricultural and Food Chemistry 52(3): 557-564

Poette J, Mekoue J, Neyraud E, Berdeaux O, Renault A, Guichard E, Genot C, Feron G (2013). Fat sensitivity in humans: oleic acid detection threshold is linked to saliva composition and oral volume. Flavour and Fragrance Journal 29(1): 39-49

Poette J, Mekoué J, Neyraud E, Berdeaux O, Renault A, Guichard E, Genot C, Feron G (2013). Fat sensitivity in humans: oleic acid detection threshold is linked to saliva composition and oral volume. Flavour Fragr. J.:

Pozo-Bayón M, Reineccius G (2009). Interactions between wine matrix macro-components and aroma compounds. In: M. V. Moreno-Arribas and P. Carmen (eds). Wine Chemistry and Biochemistry. Springer, pp 417-435

Pozo-Bayón M, Santos M, Martín-Álvarez P, Reineccius G (2009). Influence of carbonation on aroma release from liquid systems using an artificial throat and a proton transfer reaction–mass spectrometric technique (PTR–MS). Flavour and Fragrance Journal 24(5): 226–233

Rabe S, Krings U, Banavara DS, Berger RG (2002). Computerized apparatus for measuring dynamic flavor release from liquid food matrices. Journal of Agricultural and Food Chemistry 50(22): 6440-6447

Rabe S, Krings U, Berger R (2004). In vitro study of the influence of physiological parameters on dynamic in-mouth flavour release from liquids. Chemical Senses 29(2): 153-162

Roberts D, Acree T (1996). Effects of heating and cream addition on fresh raspberry aroma using a retronasal aroma simulator and gas chromatography olfactometry. J. Agric. Food Chem. 44(12): 3919–3925

Roberts DD, Acree TE (1995). Simulation of retronasal aroma using a modified headspace technique - investigating the effects of saliva, temperature, shearing, and oil on flavor release. Journal of Agricultural and Food Chemistry 43(8): 2179-2186

Robinson AL, Ebeler SE, Heymann H, Boss PK, Solomon PS, Trengove RD (2009). Interactions between wine volatile compounds and grape and wine matrix components influence aroma compound headspace partitioning. Journal of Agricultural and Food Chemistry 57(21): 10313-10322

Rodriguez-Bencomo JJ, Conde JE, Rodriguez-Delgado MA, Garcia-Montelongo F, Perez-Trujillo JP (2002). Determination of esters in dry and sweet white wines by headspace solid-phase microextraction and gas chromatography. Journal of Chromatography A 963(1-2): 213-223

Rodriguez-Bencomo JJ, Munoz-Gonzalez C, Andujar-Ortiz I, Jose Martin-Alvarez P, Victoria Moreno-Arribas M, Angeles Pozo-Bayon M (2011). Assessment of the effect of the non-volatile wine matrix on the volatility of typical wine aroma compounds by headspace solid phase microextraction/gas chromatography analysis. Journal of the Science of Food and Agriculture 91(13): 2484-2494

Sáenz-Navajas M, Campo E, Fernández-Zurbano P, Valentin D, Ferreira V (2010). An assessment of the effects of wine volatiles on the perception of taste and astringency in wine. Food Chemistry 121(4): 1139-1149

Spitaler R, Araghipour N, Mikoviny T, Wisthaler A, Via JD, Märk TD (2007). PTR-MS in enology: Advances in analytics and data analysis. International Journal of Mass Spectrometry 266(1-3): 1-7

Taylor AJ (1996). Volatile flavor release from foods during eating. Critical Reviews in Food Science and Nutrition 36(8): 765-784

Tsachaki M, Linforth RST, Taylor AJ (2005). Dynamic headspace analysis of the release of volatile organic compounds from ethanolic systems by direct APCI-MS. Journal of Agricultural and Food Chemistry 53(21): 8328-8333

van Ruth SM, Koot A, Akkermans W, Araghipour N, Rozijn M, Baltussen M, Wisthaler A, Maerk TD, Frankhuizen R (2008). Butter and butter oil classification by PTR-MS. European Food Research and Technology 227(1): 307-317

Villamor RR, Evans MA, Ross CF (2013). Effects of ethanol, tannin, and fructose concentrations on sensory properties of model red wines. American Journal of Enology and Viticulture 64(3): 342-348

Zardin E, Tyapkova O, Buettner A, Beauchamp J (2014). Performance assessment of proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOF-MS) for analysis of isobaric compounds in food-flavour applications. Lwt-Food Science and Technology 56(1): 153-160

4. 3 Impacto de parámetros relacionados con la fisiología oral en el aroma retronasal.

4. 3. 1 Influencia de la saliva considerando el efecto de la matriz del vino en la liberación del aroma retronasal.

En los apartados anteriores se comprobó el impacto de la matriz vínica durante el consumo de los vinos empleando aproximaciones *in vitro* e *in vivo*. Además estudios previos (**Publicación 3)** pusieron de manifiesto importantes diferencias interindividuales en la liberación de aroma y hacían pensar que diferencias en la fisiología del individuo durante el consumo, (como los flujos respiratorios), podrían jugar un papel en la composición del aroma retronasal, que podría condicionar por tanto la cantidad y tipos de compuestos disponibles para interaccionar con los receptores olfativos.

Entre los factores que más podrían afectar la liberación del aroma durante el consumo, la saliva ha sido de los más estudiados. Se ha comprobado que la saliva puede influir la composición del aroma retronasal a través de distintos efectos: dilución, por interacciones entre compuestos de aroma y constituyentes de la saliva, por su actividad enzimática o por su capacidad amortiguadora de pH, entre otras (Spielman 1990; Odake y col., 1998). Sin embargo, muchos de los resultados de los estudios que se han realizado para evaluar su efecto en la liberación del aroma en alimentos han llegado a conclusiones contradictorias. Por otro lado, en el caso del vino, además de la escasez de trabajos científicos encaminados a evaluar el efecto de la saliva en el aroma, se une el hecho de que los trabajos publicados presentan también resultados contradictorios. Por ejemplo, Mitropoulou y colaboradores (2011) observaron en vinos modelos suplementados con taninos y polisacáridos un aumento en la liberación de compuestos hidrofóbicos y una disminución de los compuestos más hidrofílicos en presencia de saliva. Por su parte, Genovese y colaboradores (2009) encontraron una disminución de la mayoría de compuestos volátiles en presencia de saliva en vinos blancos y tintos. Una de las posibles razones de esta divergencia en los resultados pudo ser debida a la utilización de distintas técnicas para la monitorización del aroma liberado (estáticas *vs*

dinámicas), y a las diferencias en la composición de la saliva empleada en estos estudios.

Para clarificar el papel que ejerce la saliva en la liberación del aroma del vino se requería por tanto de un estudio sistemático que evaluara el efecto tanto en condiciones dinámicas como estáticas empleado un mismo modelo experimental (vinos desorizados y reconstituidos a la misma concentración de etanol) y considerando diferencias en la composición de saliva (saliva humana, saliva artificial, controles con agua), teniendo en cuenta a su vez, el efecto de la matriz del vino (vinos blancos y tintos). Este tipo de experimento permitiría extraer conclusiones sobre el papel de la saliva en el aroma del vino, así como de los mecanismos implicados. El empleo de vinos reconstituidos aromatizados (45 compuestos) permitió además trabajar con un amplio abanico de familias químicas representativas del perfil volátil del vino.

Así, en la **Publicación 5** se evaluó el efecto de la saliva en la liberación de aroma comparando las condiciones de espacio de cabeza estáticas y dinámicas. Primero, se optimizaron las condiciones de un método de SPME de espacio de cabeza estático que se aplicó para estudiar su efecto en vinos de distinta composición (tinto joven *vs* blanco). Después, se estudió el efecto de la saliva en los mismos vinos en condiciones dinámicas y más proximas al proceso de consumo empleando un dispositivo basado en un "bio-reactor" que simulaba mejor las condiciones oro-fisiológicas durante el consumo de vino (flujos de aire, agitación). Una vez optimizadas las dos técnicas se determinó el aroma liberado por HS-SPME-GC/MS.

A continuación se presentan los resultados de este trabajo en forma de publicación científica:

Publicación 5: **Carolina Muñoz-González**, Gilles Feron, Elisabeth Guichard, Juan J. Rodríguez-Bencomo, Pedro J. Martín-Álvarez, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón. "Understanding the role of saliva on aroma release from wine by using static and dynamic headspace conditions". Publicación enviada, en fase de corrección previa publicación.

- Además este trabajo va a ser presentado como comunicación oral bajo el título "Role of saliva on wine aroma release by using in vitro static and dynamic headspace conditions" en la 3th International Conference on

Food Oral Processing, Wageningen, (the Netherlands, 29 June - 2 July, 2014. **Carolina Muñoz-González**, Gilles Feron, Elisabeth Guichard, Juan J. Rodríguez-Bencomo, Pedro J. Martín-Álvarez, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón

Publicación 5. Entendiendo el papel de la saliva sobre la liberación de aroma de vino mediante el uso de condiciones de espacio de cabeza estático y dinámico.

Understanding the role of saliva on aroma release from wine by using static and dynamic headspace conditions

Carolina Muñoz-González, Gilles Feron, Elisabeth Guichard, Juan J. Rodríguez-Bencomo, Pedro J. Martín-Álvarez, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón

Publicación enviada (bajo revision)

Abstract

The aim of this work was to determine the role of saliva on wine aroma release by using static and dynamic headspace conditions. Both methodologies were applied to reconstituted dearomatized white and red wines with different non-volatile wine matrix composition and a synthetic wine (without matrix effect). All the wines had the same ethanol concentration and were spiked with a mixture of forty five aroma compounds covering a wide range of physicochemical characteristics at typical wine concentrations. Two types of saliva (human and artificial) or control samples (water) were added to the wines. The adequacy of the two headspace methodologies for the purposes of the study (repeatability, linear ranges, determination coefficients, etc) was previously determined. After application of different chemometric analysis (ANOVA, LSD, PCA), results showed a significant effect of saliva on aroma release dependent on saliva type (differences between artificial and human) and on wine matrix using static headspace conditions. Red wines were more affected than white and synthetic wines by saliva, specifically human saliva, which provoked a reduction in aroma release for most of the assayed aroma compounds independent of their chemical structure. The application of dynamic headspace conditions using a saliva bioreactor at two different sampling points (t=0 and t=10 min) corresponding with oral (25.5 ºC) and post-oral phases (36 ºC), showed a lesser effect of saliva than matrix composition and a high influence of temperature on aroma release.

Introduction

Aroma is one of the most outstanding aspects related to food preferences and choices, especially in the case of wine, in which consumption is mainly triggered by a hedonic behavior. Therefore, aroma represents a relevant aspect in wine research and the characterization and elucidation of aroma impact compounds of different wine types has been the focus of many interesting works in this field (Guth 1997; Ferreira et al. 2002; Escudero et al. 2007). However, the retronasal aroma profile of a food during consumption might better represent the aroma fraction involved in the interaction with the olfactory receptors than the orthonasal aroma profile, therefore, it should be more closely related with aroma perception (Pierce and Halpern 1996).

In the case of the consumption of liquid foods, such as wine, retronasal aroma is produced by the breathing airflow after swallowing sweeping the aroma molecules retained in the oral or throat cavities travelling via the nasopharynx from the mouth or throat to the nose (Taylor 1996; Buettner et al. 2001; Buettner et al. 2002). It has been shown that orthonasal (odor sense when smelling a food) and retronasal aroma perception can be different (Burdach et al. 1984; Voirol and Daget 1986; Linforth et al. 2002). Different factors involved in the intra-oral release of aroma compounds during consumption (saliva, interaction with mucosa, temperature, breathing flows, in-mouth air cavity volumes, change, etc.) seem to be related to these differences (Burdach and Doty 1987; Overbosch et al. 1991; Taylor 1996; Harrison 1998; Buettner and Schieberle 2000; Buettner and Beauchamp 2010; Mishellany-Dutour et al. 2012).

Saliva is a complex dilute aqueous solution in which its composition varies depending on the respective physiological status, types of food consumed, oral hygiene, etc (Neyraud et al. 2012). Saliva contains numerous inorganic salts (sodium, calcium, potassium, chloride, phosphate and bicarbonate) (Drobitch and Svensson 1992) and organic components such as enzymes (amylase, lipases, proteases, etc.) (Buettner 2002; Buettner 2002; Neyraud et al. 2012) and proteins (mucins, proline rich proteins, histidine rich proteins, etc.) (McRae and Kennedy 2011; Salles et al. 2011). Previous studies have shown that saliva might exert an important role on aroma release through different physicochemical (dilution of aroma due to the aqueous phase of saliva, changes in the pH of the food, hydration of the food which favors aroma release, interaction with salts causing a salting out effect, interaction with proteins); chemical

(degradation of odorants); biochemical (degradation of odorant or release from aroma precursors), or even physiological effects (impact on velum-tongue seal formation and swallowing performance), which form part of many previous works performed on this topic(vanRuth et al. 1996; Friel and Taylor 2001; Buettner 2002; Buettner 2002; van Ruth and Buhr 2003).

Nonetheless, many of the studies performed on the saliva effect on aroma release in simple and real food systems seem to be contradictory. Some studies have shown that saliva reduces aroma release: e.g. in pectin gels (Hansson et al. 2003) bell peppers (van Ruth and Buhr 2003) or French beans (vanRuth et al. 1996); whilst others have shown an increase in volatiles released from model gels (Boland et al. 2004)or primary and multilayer oil/water emulsions (Benjamin et al. 2012); even though, there are works showing the lack of effect of saliva on aroma release: e.g. in model cheeses (Pionnier et al. 2004) and from starch and water liquid systems (Rabe et al. 2004).

Undoubtedly, the physicochemical characteristics of the volatile compounds are outstanding parameters in determining the degree of interaction with saliva components (Salles et al. 2011). In addition, saliva might induce an array of processes with sometimes opposite effects on aroma release and perception. Therefore, the overall impact of saliva needs to be specifically studied for each food system and aroma composition. Moreover, in many of the above mentioned works, different types of saliva had been used (human, artificial saliva with different compositions), therefore, a comparison of the effect of saliva performed in such different conditions is not straightforward.

As stated in a recent review on wine aroma analysis, the number of studies regarding aroma release during wine consumption using *in vitro* or *in vivo* approaches is scarce (Munoz-Gonzalez et al. 2011), and research on the role of different intra-oral factors (such as saliva) which might be involved in aroma release during wine drinking is still incipient. The effect of saliva has been mainly studied because of its involvement in wine astringency (Kallithraka et al. 2001; Simon et al. 2003; Mateus et al. 2004; Cala et al. 2012; de Freitas and Mateus 2012; Rinaldi et al. 2012). However, there are very few studies focused on the role of saliva on wine aroma release (Genovese et al. 2009; Mitropoulou et al. 2011). Although the relatively short-intra-oral period of consumption of liquid foods, could indicate a limited effect of saliva on aroma release, the formation

of intra-oral (and pharyngeal) aroma depots (Buettner et al. 2001) and the fact that natural swallowing of saliva is continuously performed, makes the idea that saliva might exert an important role in the perception of wine aroma during consumption perfectly viable, but also affecting the persistence of aroma perception during the post-oral phase of wine consumption. Very recently, it was shown that enzymatic degradation of palm wine odorants in the presence of saliva was not noticeable among pyrazines, pyrrolines and most alcohols but was quite pronounced among aldehydes, esters and thiols (Lasekan 2013).

Likewise, in other food systems, the few studies concerning the effect of saliva on aroma release from wines are contradictory. In the work of Genovese and collaborators (Genovese et al. 2009), saliva induced, in general, a decrease on aroma release for most of the wine volatiles, and this effect seemed to be more important in white than in red wines. On the contrary, Mitropoulou and co-workers (Mitropoulou et al. 2011), observed an enhancement on the release of hydrophobic compounds from model wines and a decrease in the release of the most hydrophilic compounds in the presence of saliva, although this effect was dependent on the concentration of tannins and polysaccharides. Both works were, however, performed in very different conditions; by using dynamic conditions in the work by Genovese et al., (Genovese et al. 2009), and by using a static headspace approach in the work of Mitropoulou et al., (Mitropoulou et al. 2011). The dynamic conditions are advisable to achieve more realistic conditions to that accounting for during food consumption, however, the static conditions have been shown to be better suited for the study of interacting effects that otherwise might be underestimated with the first approach (Friel and Taylor 2001; Fabre et al. 2002).

Therefore, the aim of this work was to determine the role of saliva on wine aroma release by using both static and dynamic headspace conditions. In an attempt to follow a systematic study, avoiding the influence of different factors other than those of interest in this work (saliva effect and wine type), both methodologies were applied to reconstituted wines (with different non-volatile wine matrix composition) and a synthetic wine (with no matrix effect) keeping the concentration of ethanol and aroma compounds the same. In addition, two types of saliva (human and artificial) and control samples (with water) were used to better understand the different mechanisms that saliva might induce on the release of aroma compounds from wine.

Material and methods

Wine samples

Two commercial Spanish wines representative of different wine matrix compositions were selected for this study: a young Verdejo white wine (W-wine), and a young Tempranillo red wine (R-wine).

Reconstituted wines

Deodorization procedure

Wines were deodorized with Amberlite XAD-2 (Supelco, Bellefonte, PA, USA). Two 25 cm-length glass columns (Pobel, Madrid, Spain), one for each wine type, filled with 100 g of Amberlite XAD-2 were prepared by sequentially conditioning with 250 mL of dichloromethane, then methanol and finally 375 mL of a 12 % (v:v) hydroethanolic solution. After this, wine samples were filtered through glass wool and loaded into the column by slowly passing 750 mL of each wine.

Deodorized wines (750 mL of each) were transferred to 250 mL vials and were completely dried in a lyophiliser (Labconco, Kansas City, MO, USA). Five samples per wine type were prepared using this procedure. To replace the oxygen from the samples, all the dry samples were exposed to a Nitrogen atmosphere and stored at 4 ºC until sample preparation. This procedure practically ensured the complete elimination of the original aroma compounds in the wines as was confirmed by HS-SPME-GC-MS analysis.

Wine reconstitution

Deodorized wines were reconstituted with a hydroalcoholic solution and spiked with a volatile mixture to a final ethanol concentration of 12%. This aroma mixture composed of 45 aroma compounds (manufacturers: Aldrich, Fluka, Merck, Firmenich, Lancaster and Scharlau) representative of a typical wine aroma profile to produce the final concentration of each aroma compound shown in **Table 1**. This table also shows some of the typical gas chromatography and physicochemical properties of these compounds.

Table 1. Chromatographic and physicochemical characteristics of the volatile compounds employed in this study.

Nº	Compound	RI exp[a]	RI lit[b]	Ion Q[c] (m/z)	MW[d] (g/mol)	Log*P*[e]	BP[f] (ºC)	DESCRIPTOR[g]	CAS number	Concentration[h] (mg/L)
1	Ethyl Propanoate	< 1000	950	57	102	1.2	99.1	fruit	105-37-3	0.61
2	Isobutyl acetate	1018	1018	56	116	1.8	116.5	fruit, apple, banana	110-19-0	0.33
3	α-pinene	1030	1035	93	136	4.8	156.0	pine, turpentine	80-56-8	0.20
4	Ethyl butanoate	1043	1040	71	116	1.9	121.5	apple	105-54-4	0.54
5	Ethyl 2-methylbutanoate	1060	1056	57	130	2.3	133.0	apple	7452-79-1	0.29
6	Butyl acetate	1079	1079	43	116	1.8	126.1	pear	123-86-4	0.35
7	Isobutanol	1100	1103	74	74	0.8	108.0	wine, solvent, bitter	78-83-1	1.38
8	β-pinene	1120	1118	93	136	4.4	164.0	pine, resin, turpentine	127-91-3	0.25
9	Isoamyl acetate	1131	1117	70	130	2.3	142.5	banana	123-92-2	0.69
10	1-butanol	1154	1145	56	74	0.8	117.0	medicine, fruit	71-36-3	0.93
11	Limonene	1217	1208	68	136	4.8	176.0	lemon, orange, citrus	5989-27-5	0.23
12	Isoamylic alcohols	1217	1208	55	86	1.3	128.0	wine,onion	123-51-3	30.01
13	Ethyl hexanoate	1247	1231	88	136	2.8	167.0	apple peel, fruit	123-66-0	0.89
14	Hexyl acetate	1286	1276	56	144	2.8	171.5	fruit, herb	142-92-7	0.92
15	1-Hexanol	1364	1362	56	102	2.0	156.0	resin, flower, green	111-27-3	0.91
16	*trans*-3-Hexen-1-ol	1376	1386	67	100	1.6	156.5	mosss, fresh	928-97-2	0.31
17	*cis*-3-Hexen-1-ol	1399	1398	67	100	1.6	156.5	grass	928-96-1	0.33
18	Ethyl octanoate	1453	1444	127	172	3.8	208.5	fruit, fat	106-32-1	0.79
19	Furfural	1487	1466	95	96	0.4	161.7	bread, almond, sweet	98-01-1	0.85
20	Linalool	1557	1544	93	154	3.0	198.0	flower, lavender	78-70-6	0.24
21	5-Methylfurfural	1603	1573	109+110	110	0.7	187.0	almond, caramel, burnt	620-02-0	0.54
22	Terpinen-4-ol	1633	1606	93	154	3.3	209.0	turpentine, nutmeg, must	2438-10-0	0.30
23	Ethyl decanoate	1658	1636	101	200	4.8	241.5	grape	110-38-3	0.38
24	Furfuryl alcohol	1677	1672	98	98	0.3	171.0	burnt	98-00-0	0.55
25	γ-butyrolactone	1674	1647	42	86	-0.6	204.0	caramel, sweet	96-48-0	1.97
26	Diethyl succinate	1693	1647	101	174	1.2	217.7	wine, fruit	123-25-1	0.69

27	α-Terpineol	1725	1688	59	154	3.0	217.5	oil, anise, mint	10482-56-1	0.20
28	β-Citronellol	1780	1768	69	156	3.9	224.0	rose	106-22-9	0.28
29	Nerol	1820	1792	69	154	3.6	225.0	sweet	106-25-2	0.23
30	β-phenylethyl acetate	1852	1829	104	164	2.3	232.6	rose, honey, tobacco	103-45-7	0.74
31	Ethyl dodecanoate	1860	1842	88	228	5.7	281.2	leaf	106-33-2	0.43
32	β-Damascenone	1860	1815	190	190	4.2	275.0	apple, rose, honey	23726-93-4	0.20
33	α-ionone	1894	1840	93	192	3.9	259.5	wood, violet	127-41-3	0.10
34	Hexanoic acid	1900	1829	60	116	2.1	203.0	sweat	142-62-1	0.83
35	Benzyl alcohol	1909	1897	79	108	1.1	205.3	sweet, flower	100-51-6	0.74
36	*trans*-whiskey lactone	1935	1977	99	156	2.0	260.6	flower, lactone	80041-01-6	0.69
37	β-phenylethyl alcohol	1948	1925	91	122	1.4	218.2	honey, spice, rose, lilac	60-12-8	3.28
38	β-ionone	1985	1912	177	192	3.8	262.9	raspberry, violet,	79-77-6	0.10
39	*cis*-whiskey lactone	2010	1985	99	156	2.0	260.6	flower, coconut	80041-00-5	0.69
40	4-ethylguaicol	2067	2031	137	152	2.4	248.39	spice, clove	2785-89-9	0.35
41	γ-Nonalactone	2081	2042	85*	156	2.1	243.0	coconut, peach	104-61-0	0.17
42	Octanoic acid	2107	2083	60	144	3.1	239.0	sweat, cheese	124-07-2	1.96
43	Eugenol	2205	2164	164*	164	2.3	253.2	clove, honey	97-53-0	0.21
44	4-Ethylphenol	2205	2170	107*	122	2.6	217.9	must	123-07-9	0.40
45	Decanoic acid	2328	2361	60	172	4.1	278.6	rancid, fat	334-48-5	0.78

[a] Experimental retention index calculated with an alkane mixture (C5–C30) on DB-WAX column.
[b] Linear retention index from literature (NIST Chemistry Webbook).
[c] Ion of quantification (* Compound determined in SIM mode).
[d] Molecular weight.
[e] Hydrophobic constant estimated using molecular modeling software EPI Suite (U.S. EPA 2000-2007).
[f] Boiling point estimated using molecular modeling software EPI Suite (U.S. EPA 2000-2007).
[g] From Flavornet (http://www.flavornet.org; accessed October 2009) database, from NIST web chemistry book (2005) (http://www.webbook.nis.gov/chemistry).
[h] Final concentration in the wine.

As well as the two types of reconstituted wine matrix, a synthetic wine (S-wine) representing a sample with 'no matrix effect' was prepared by mixing an hydroalcoholic solution with 4 g/L tartaric acid (Panreac, Barcelona, Spain) and adjusting the pH to 3.5 with NaOH (Panreac).

The influence of ethanol on the volatility of aroma compounds was not considered in this study, since it has been extensively demonstrated (Conner et al. 1998; Escalona et al. 1999; Rodriguez-Bencomo et al. 2002; Robinson et al. 2009). Therefore, ethanol was kept at the same concentration in all reconstituted and synthetic wines.

Human saliva

Stimulated human saliva was collected from 20 volunteers as described before (Poette et al. 2013). Participants could not consume food and water one hour before sampling. To stimulate production, volunteers chewed a little piece of Parafilm™ and spat out as much saliva in a bottle as they could. Sodium azide (NaN_3, Sigma-Aldrich, Saint Quentin Fallavier, France) was added at a final concentration of 0.02% to avoid bacteria and fungi contamination and development. To obtain most representative salivary composition, the different saliva samples were pooled, mixed and centrifuged at 15000 G for 15 min. After that, the salivary pool was filtered through a 0.22 µm Sartorius device under vacuum at 4ºC, to remove saliva bacteria. Finally, saliva was sampled into pots of 20 mL and stored at -80 ºC until use.

Artificial saliva

Artificial saliva was prepared as previously described (vanRuth et al. 1996) by dissolving in 1 L of water (purified by a Milli-Q system) 5.028 g $NaHCO_3$, 1.369 g K_2HPO_4 x 3 H_2O, 0.877 g NaCl, 0.477 KCl, 0.441 g $CaCl_2$ x 2 H_2O and 2.16 g mucin (type 1-S from bovine submaxillary glands) from Sigma, (Milan, Italy). The artificial saliva was stored at 4ºC until use.

Static Headspace-SPME sampling procedure

In the human mouth, the average ratio liquid food/saliva had previously been shown to be 5/1 w/v (Genovese et al., 2009). Therefore, blends containing the reconstituted white and red wines (W-wine, R-wine) or the synthetic wine (S-wine) were prepared by adding ten mL of the wines spiked with the volatile mixture in a 20

mL vial (Agilent Technologies, Palo Alto, CA, USA). After that, 2 mL of water, human or artificial saliva were added. The headspace vials were immediately closed with a screw cap and polytetrafluoroethylene (PTFE)/silicone septum (Supelco, Bellefonte, PA, USA) and were placed in the incubator of an automatic headspace sampling device (GERSTEL MPS 2, Gerstel Inc., Mülheim an der Ruhr, Germany) at 11 ºC. The wine:saliva mixture was previously pre-incubated for 12 min at 36 ºC. In the control wine, the extraction was performed in the headspace of each vial at different incubation times (5, 15, 30 and 45 minutes) to follow the kinetic of aroma release and to determine the equilibrium time, using a DVB/CAR/PDMS (Divinylbenzene/Carboxen/Polydimethylsiloxane 50/30 µm thickness -2 cm length-) coated SPME fiber (Supelco, Bellefonte, PA). After the incubation time the fiber was exposed to the headspace above the sample for 2 min, and the vial was maintained at 36 °C. Desorption was performed in the injector of the GC system (Agilent 6890N) in splitless mode for 1.5 min at 270 ºC. After each injection the fiber was cleaned for 30 min to avoid any memory effect. Each analysis was performed in triplicate (one injection per sample vial). Linearity and reproducibility of the procedure were previously determined by using a synthetic wine spiked with different amounts of the aroma solution (75, 150 and 300 µL) covering as closely as possible the wine aroma concentration expected in wines (Rodriguez-Bencomo et al., 2011).

The results of this study are shown in supplementary **Table 1 of the Supporting information**. Herein, satisfactory values for the regression coefficients for most of the aroma compounds were obtained, which ranged from 0.910 to 1.000 and the regression RSDs were also acceptable, with values lower than 20% (except for γ-butyrolactone and ethyl dodecanoate). These results confirmed the lack of interactions between individual volatile aroma compounds in the mixture at the concentrations used(Lubbers et al. 2004), confirming the adequacy of the technique to perform this study.

Table 1(Supporting information). Linear ranges and regression parameters (slope, coefficient of determination (R^2), and reproducibility (CV)) calculated for the aroma compounds by using static and dynamic (t=0 and t=10 minutes) HS-SPME-GC/MS analysis.

	Static HS				Dynamic HS (t=0)				Dynamic HS (t=10)			
	Linear range	**Slope**	**R^2**	**CV (%)**	**Linear range (mg/L)**	**Slope**	**R^2**	**CV (%)**	**Linear range**	**Slope**	**R^2**	**CV (%)**
Ethyl Propanoate	0,37-0,73	242172	0,994	8,5	0,49-1,46	403856	0,998	4,7	0,49-1,46	340762	0,997	6,7
Isobutyl acetate	0,19-0,39	282947	0,995	7,9	0,26-0,78	392292	0,997	6,0	0,26-0,78	387509	0,998	5,7
α-pinene	0,12-0,23	24293300	0,995	7,9	0,16-0,47	1858600	0,959	22,3	0,16-0,94	1352830	0,984	15,2
Ethyl butanoate	0,32-0,65	609583	0,994	8,5	0,43-1,30	785951	0,996	7,0	0,43-1,30	858621	0,999	4,2
Ethyl 2-methylbutanoate	0,17-0,35	791392	0,999	4,5	0,23-0,70	793989	0,983	14,8	0,23-0,70	899014	0,997	6,5
Butyl acetate	0,21-0,41	842904	0,999	3,3	0,28-0,83	887148	0,997	6,1	0,28-0,83	1072520	0,998	4,7
Isobutanol	0,83-3,31	653	0,978	16,7	1,10-3,31	965	0,993	7,2	1,10-3,31	1111	0,996	7,1
β-pinene	0,15-0,30	20770200	0,997	6,7	0,20-0,61	2442810	0,971	18,9	0,20-1,21	1925700	0,988	13,0
Isoamyl acetate	0,41-0,82	912097	0,999	4,2	0,55-1,64	968701	0,993	9,1	0,55-1,64	1147780	0,998	4,1
1-butanol	0,56-2,23	7714	0,975	17,9	0,74-4,45	7679	0,982	15,6	0,74-2,23	13136	0,990	8,7
Limonene	0,14-0,27	3322440	0,993	10,2	0,18-1,09	444733	0,979	17,4	0,18-1,09	465258	0,994	9,3
Isoamylic alcohols	18,01-36,01	16912	0,995	8,3	24,01-144,04	10032	0,973	18,7	24,01-72,02	19087	0,994	7,0
Ethyl hexanoate	0,53-1,07	1772110	0,998	5,8	0,71-2,13	1376330	0,994	8,9	0,71-2,13	185835	0,996	5,3
Hexyl acetate	0,55-1,11	1059920	0,997	6,1	0,74-2,21	802331	0,994	8,7	0,74-2,214	1116980	0,997	4,6
1-Hexanol	0,55-1,09	83308	1,000	1,7	0,73-4,37	56964	0,996	7,2	0,73-2,18	125277	0,999	3,2
trans-3-Hexen-1-ol	0,18-0,37	27311	1,000	2,7	0,25-1,48	17410	0,985	14,4	0,25-1,48	35190	0,981	15,9
cis-3-Hexen-1-ol	0,20-0,40	27776	1,000	2,0	0,26-1,58	16264	0,997	7,1	0,26-1,58	34055	0,999	4,2
Ethyl octanoate	0,47-1,88	812081	0,992	10,4	0,63-3,77	610626	0,996	7,5	0,63-3,77	849454	0,994	9,3
Furfural	0,51-2,04	30037	1,000	2,1	0,68-4,08	18419	0,995	8,6	0,68-4,08	39829	0,998	5,8
Linalool	0,14-0,57	80270	0,999	4,5	0,19-1,14	53555	0,996	8,0	0,19-1,14	132218	0,999	4,0
5-Methylfurfural	0,33-1,30	20442	0,999	2,6	0,43-2,60	11986	0,996	7,6	0,43-2,60	25844	0,998	5,8
Terpinen-4-ol	0,18-0,72	50774	0,999	4,1	0,24-1,44	26965	0,996	8,2	0,24-1,44	74167	0,999	4,1
Ethyl decanoate	0,23-0,92	1938790	0,997	6,2	0,31-1,84	705095	0,993	10,5	0,31-1,84	1205770	0,990	12,3
γ-butyrolactone	1,18-4,73	645	0,910	39,7	1,58-9,46	444	0,897	35,0	1,58-9,46	638	0,941	26,5
Furfuryl alcohol	0,33-1,33	2368	0,977	17,1	0,44-2,65	1192	0,977	17,6	0,44-2,65	1948	0,990	12,0

Diethyl succinate	0,41-1,65	20124	0,999	3,0	0,55-3,30	9036	0,996	7,8	0,55-3,30	20278	0,998	5,7
α-Terpineol	0,12-0,49	48704	0,998	5,2	0,16-0,97	21457	0,993	10,0	0,16-0,97	56086	0,998	5,7
β-Citronellol	0,17-0,68	67146	0,999	4,0	0,23-1,36	36139	0,994	9,5	0,23-1,36	103191	0,997	6,8
Nerol	0,14-0,55	61926	0,999	4,5	0,18-1,09	29815	0,996	8,2	0,18-1,09	73761	0,994	10,2
β-phenylethyl acetate	0,44-1,78	157572	0,999	2,7	0,59-3,55	111758	0,998	7,8	0,59-3,55	231698	0,998	5,9
Ethyl dodecanoate	0,26-1,02	4746570	0,958	22,8	0,34-2,04	284811	0,918	32,5	0,34-2,04	708281	0,977	18,8
β-Damascenone	0,20-0,48	64663	0,998	5,4	0,16-0,95	52072	0,996	8,1	0,16-0,95	126982	0,999	4,4
α-ionone	0,06-0,23	209245	0,998	4,7	0,08-0,46	163821	0,995	9,1	0,08-0,46	407739	0,998	5,6
Hexanoic acid	0,49-1,98	10433	0,985	14,3	0,66-3,96	2695	0,945	27,7	0,66-3,96	4811	0,987	14,2
Benzyl alcohol	0,44-1,76	4349	0,983	14,9	0,59-3,53	2068	0,985	14,5	0,59-3,53	3497	0,989	11,9
trans-whiskey lactone	0,41-1,66	11247	0,997	6,5	0,55-3,31	5591	0,995	8,4	0,55-3,31	12325	0,999	4,6
β-phenylethyl alcohol	1,97-7,87	88918	0,993	9,8	2,62-15,74	3935	0,996	7,8	2,62-15,74	7202	0,997	6,8
β-ionone	0,06-0,25	325244	0,999	4,1	0,08-0,50	236677	0,993	11,0	0,08-0,50	600232	0,996	7,6
cis-whiskey lactone	0,41-1,66	7947	0,994	8,9	0,55-3,31	3836	0,996	7,7	0,55-3,31	8348	0,998	5,9
4-ethylguaicol	0,21-0,85	39110	0,999	3,8	0,28-1,69	20339	0,998	5,7	0,28-1,69	40694	0,996	8,0
γ-Nonalactone	0,10-0,42	20501	0,984	14,6	0,14-0,83	9234	0,989	12,5	0,14-0,83	18427	0,993	10,1
Octanoic acid	1,18-4,71	13347	0,973	18,8	1,57-9,42	2784	0,979	18,5	1,57-9,42	5864	0,975	21,0
Eugenol	0,13-0,51	10936	0,996	7,8	0,17-0,51	6603	0,989	11,9	0,17-1,03	9168	0,987	13,7
4-Ethylphenol	0,24-0,96	25666	0,993	9,8	0,32-1,91	11984	0,997	7,2	0,32-1,91	22706	0,993	10,5
Decanoic acid	0,47-1,87	30595	0,969	19,9	nd	nd	nd	nd	nd	nd	nd	nd

CV (%) = (s/$\bar{y}$) x 100, residual standard deviation expressed as a percentage of the mean value.

Dynamic Headspace-SPME sampling procedure

A saliva bioreactor cell was used for these assays (Poette et al. 2010). This device was specifically designed to evaluate the particular role of saliva during liquid and semi-solid food consumption. It was composed of a water-jacketed glass flask (100 ml), which allowed a temperature control of the sample set at 36 ºC. This device has five orifices. The first permits clean air to enter the flask to purge the sample (100 mL/min), therefore, reproducing the dynamic conditions of the breathing phenomena. A second orifice is the purge gas outlet, which is connected through a heated transfer line to a flowmeter. In the third orifice the SPME fiber is inserted and the fourth opening is where the sample is introduced. Finally, in order to mix the sample as what might occur in the mouth, a fifth orifice allowed the introduction of a stir bar with digital speed control. An agitation rate of 150 rpm was employed. This last orifice was firmly sealed around the stir bar shaft with a septum to avoid leaks from the flask. During the experiment setup, the sample was added to the apparatus using a glass funnel.

Following the above mentioned 5/1 average ratio liquid food/saliva in the human mouth, 10 ml of water, human saliva or artificial saliva were transferred into the sample flask (100 ml) which was kept at 36 ºC, and then 50 mL of wine were then added. The headspace was continuously flushed with purified Nitrogen gas (100 mL/min). Even if the experimental conditions were not directly comparable with conditions in the mouth, two sampling points were assayed to analyze the aroma release resulting from the incubation of control, red and white wines in contact with water, human saliva or artificial saliva (**Figure 1**). The first one, corresponding to an initial sampling time (t=0 min), in which the saliva/wine mixture temperature raised from 25.5 ºC to 32.3 ºC that might correspond with the introduction of the sample in the mouth (oral-phase). The second sampling point (t=10 min at 36 °C) was more related to the post-oral phase in which aroma from the remaining wine sample could be released within the oral cavity at physiological temperature. In both cases, extraction was performed for 2 minutes. Two or three replicates for each sample type were analyzed depending on the experiment.

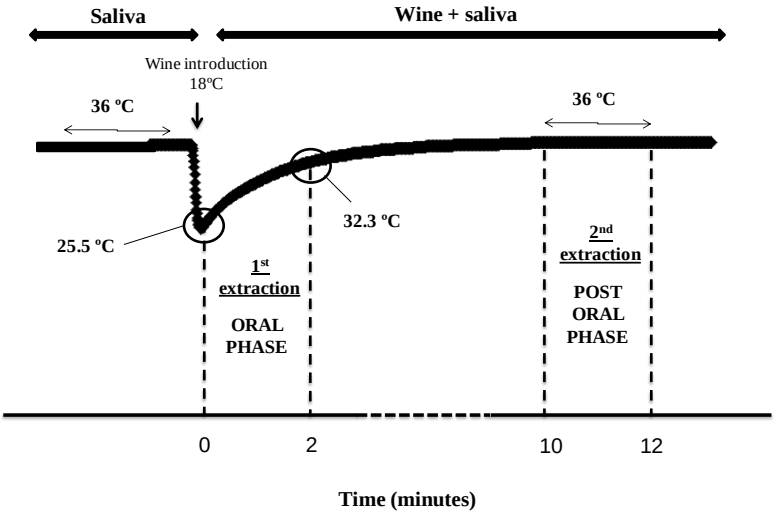


Figure 1. Representation of the sampling procedure employed during the dynamic HS-SPME-GCMS analysis.

It has been shown that inter-fiber repeatability is worse than the intra-fiber accuracy (Popp and Paschke 1997; Natera Marin et al. 2002; Yang et al. 2002). Therefore, a preliminary inter-fiber repeatability study was performed in order to select the most similar fibers to complete the study. For this study nine SPME fibers were used to recover the 45 aroma compounds of the aroma mixture added to synthetic wines, and the two SPME fibers exhibiting the lowest variation (less than 10 % RSDs for the extraction of the same aroma compound) were selected and used for the complete set of experiments.

In addition, because the dynamic HS-SPME sampling approach is based in a non-equilibrium situation, a linearity study was carried out in order to seek the relationship between the adsorbed amount of volatiles on the fiber and their initial concentration in the sample. To do so, a synthetic wine spiked with four different amounts of the aroma mixture, was submitted to the dynamic HS extraction conditions as explained above. These results are shown in the supplementary table 1 in the supporting information. As it can be seen there is good linearity, high coefficients of determination (R^2) (better than 0.9 in the assayed concentration range, except for γ-butyrolactone) and adequate regression RSDs for most of the assayed compounds independent of the time at which sampling was performed (0 and 10 minutes). The lack of fit test also showed the adequacy of the propose regression models (p values > 0.01 for most of the aroma compounds) (data not shown). Therefore, the adsorbed amount of aroma compounds in the SPME was linearly proportional to their initial concentration

in the sample matrix, highlighting the adequacy of the technique for quantification purposes, which is in agreement with other theoretical and experimental studies performed in simpler aroma systems(Ai 1997).

GC-MS analysis

The identification of volatile compounds was carried out with a Gas Chromatograph Agilent 6890N coupled to a quadrupole Mass Detector Agilent 5973. After desorption of the SPME fiber (270 ºC, splitless), volatile compounds were separated on a DB-Wax polar capillary column (60 m × 0.25 mm i.d. × 0.50 µm film thickness) from Agilent (J&W Scientific, Folsom, USA). Helium was the carrier gas at a flow rate of 1 mL/min. The oven temperature was initially held at 40 ºC for 5 min, then increased at 4 ºC/min to 240 ºC and held for 20 min.

For the MS system (Agilent 5973N), the temperatures of the transfer line, quadrupole and ion source were 250, 150 and 230 ºC respectively. Electron impact mass spectra were recorded at 70 eV ionization voltages and the ionization current was 10 µA. The acquisitions were performed in Scan (from 35 to 350 amu) and SIM modes for some specific compounds as indicated in **Table 1**.

The identification of compounds was based on their retention indexes (RIs), comparison of retention times and mass spectra. RIs were calculated from the retention times of n-alkanes (C5–C30) on the same column. RIs values were compared with RIs from compilations (NIST Chemistry Webbook. 2010. Available: http://webbook.nist.gov/ [2012]) or from the literature. The mass spectra were compared with those from three databases: NIST 2.0, WILEY 138 and INRAMASS (internal database achieved using standard compounds).

To avoid possible wine matrix interaction phenomena (Rodriguez-Bencomo et al. 2011) instead of using an internal standard compound, release data were referred to absolute peak area, once the precision of the data was proven.

Chemical wine matrix composition

Total acidity and pH, total polyphenols, neutral polysaccharides, residual sugar and nitrogen compounds (total nitrogen, free amino acids and peptides) were

determined following previously described analytical procedures (Rodriguez-Bencomo et al. 2011).

Saliva biochemical analysis

Protein concentration

The protein concentration was determined using Bradford protein assay Quick Start (Bio-Rad, France) with gamma-globulin as the standard for calibration.

Enzymatic activities

Lipolysis, proteolysis, lysozyme and amylase activities were measured as previously described (Neyraud et al. 2012; Poette et al. 2013).

Statistical analysis

Aroma release data (absolute peak area) were submitted to two-way ANOVA to determine significant effects of the studied factors (saliva type and wine type). In addition, for each aroma compound and wine type (red, white and synthetic) differences between medium type (with human saliva, artificial saliva and water) were subsequently examined by least significant difference (LSD) test. The significance level was P=0.05 throughout the study. Principal component analysis (PCA) was also applied to examine the relationship between aroma release data and wine samples. The STATISTICA program for Windows version 7.1 was used for data processing (StatSoft, Inc., 2005, www.statsoft.com). Linear regression analysis to establish the calibration curves of each aroma compound and the lack of fit test to judge the adequacy of the models were performed by using the Statgraphics Centurion XV Version 15.2 (Manugistics, Rockville, MD, USA).

Results and discussion

To understand the effect of saliva composition on the release of aroma compounds, two types of wines, a white and a red wine were previously deodorized, reconstituted to the same ethanol content and aromatized at the same concentration with the aroma mixture (**Table 1)**. With this procedure, it was guaranteed that ethanol did not affect the partition of volatile compounds into the headspace and that both wine matrices had the same concentration of aroma compounds. Therefore, the main

differences between both wines were exclusively due to their matrix composition. **Table 2** shows the chemical composition of both reconstituted wines. The percentage of non-volatile residue and the pH values were very similar. The non-volatile residue was 2.17% (w/w) and 2.99 % (w/w) and the pH was 3.23 and 3.79 for the white and red wines respectively. Total acidity was slightly lower for the red wine (4.29 mg tartaric acid/L) compared to 5.66 mg tartaric acid/L in the case of white wine. The major differences were however, in the total polyphenol content, neutral polysaccharides, residual sugars and nitrogen containing compounds (amino acids and peptides) that were significantly higher in the red wine. These differences in matrix composition have been previously shown to affect the release of aroma compounds in static conditions (Rodriguez-Bencomo et al. 2011). In addition to this, a synthetic wine with the same ethanol concentration and pH = 3.5 that could be considered as a wine with "no matrix" effect was also prepared.

Table 2. Chemical composition of the red and white wines employed in this study**.**

	White Wine		**Red Wine**	
	Mean	SD	Mean	SD
Non-volatile residue (% w/w)	2.17	0.11	2.99	0.08
pH	3.23	0.01	3.79	0.01
Total acidity (mg tartaric acid/L)	5.66	0.1	4.29	0.2
Total polyphenols (mg gallic acid/L)	269.95	17.2	1647.98	292.8
Neutral polysaccharides (g mannose/L)	1.67	0.5	2.50	0.9
Residual sugars (g/L)	1.12	0.2	3.68	0.5
Total nitrogen (mg/L)	239.96	32.9	406.00	65.7
Amino acids + peptides (mg N/L)	49.54	2.2	133.51	10.9
Amino acids (mg N/L)	30.67	0.8	58.57	1.4
Peptides (mg N/L)*	18.87	-	79.94	-

Values are average of two determinations except for pH (average of three determinations).* This value is indirectly determined as the difference between the analytical determination of amino acids plus peptides and free amino acids, therefore SD (Standard deviation) values are not included in the table.

For the saliva experiments, two types of saliva were used, artificial saliva with mucin prepared in agreement with the recipe previously described and human saliva collected from different volunteers and mixed together to form a single pool. The composition, regarding total protein content and enzymes (amylase, lipase, lysozyme and protease) was analyzed. The major enzymatic activity detected in the human saliva

was lysozyme (698.06 U/mL) followed by proteolysis (16.77 U/mL) and amylase (8.01 U/mL) and in a lesser extent lipase (0.95 mU/mL). These values are in the same order of magnitude to those previously published (Neyraud et al. 2012; Mounayar et al. 2013; Poette et al. 2013) except for proteolysis activity, which was higher in our study. In addition to the two types of saliva, control experiments were also performed by adding the same amount of water instead of saliva. With this control, we also eliminated the dilution effect exerted by saliva on volatile release, which has also been described (van Ruth et al. 2001; Benjamin et al. 2012). In addition, this type of experiment could provide us important information regarding whether saliva enzymes might have an impact on aroma release from wine as it has been previously shown in simple aroma/saliva mixtures (Hussein et al. 1983; Buettner 2002; Buettner 2002).

Effect of saliva on aroma release using static headspace conditions

Although static headspace conditions do not mimic the dynamic conditions accounting for during drinking or eating, this technique has been largely used to study aroma interactions with food matrix components to determine their effect on aroma release (Friel and Taylor 2001; Kopjar et al. 2010; Mitropoulou et al. 2011; Rodriguez-Bencomo et al. 2011). Even so, different authors have shown that this is a reliable approach to investigate partition in more controlled and simple conditions, which allows us to envisage this subtle phenomena with importance on aroma release, that otherwise might be underestimated by using dynamic HS methods (Friel and Taylor 2001; Fabre et al. 2002).

In this work, the aroma release behavior of a mixture of forty five volatile compounds characteristic of the wine aroma profile and with very different physicochemical characteristics (**Table 1**) was evaluated in presence and absence of human and artificial saliva by using a previously validated static HS-SPME approach (see **Table 1 in supporting information**). Preliminary experiments were performed in order to determine the equilibration time (5, 15, 30, 45 minutes) for most volatiles of the aroma mixture. From the analysis of the kinetic profiles it was found that five minutes of incubation was enough for the equilibration of most of the aroma compounds of the mixture. Only ten of them (ethyl propanoate, isobutyl acetate, isobutanol, isoamyl acetate, 1-butanol, ethyl octanoate, furfuryl alcohol, α-terpineol, benzyl alcohol and decanoic acid) were not equilibrated after 5 minutes. Nonetheless, since the main

objective of this work was to compare wine samples performed under identical experimental conditions, this should not be a constraint for the validity of the data and five minutes was adopted as the sampling time to perform the experiment, which are closer conditions to real physiological situations.

Data corresponding to absolute peak areas of the aroma compounds determined by HS-SPME-GC-MS analysis in the three types of wines (white, red and synthetic) incubated with the two types of saliva (artificial and human) and water, were submitted to a two-factorial ANOVA to determine the magnitude of the effect of matrix composition and type of saliva on aroma release. Results of this analysis showed that both effects and the interactions (matrix composition *x* type of saliva) significantly affected the majority of aroma compounds. From a total of forty five aroma compounds, thirty seven were affected by the type of saliva and thirty three by matrix composition (data not shown). This showed the similar importance of both factors on aroma release in static headspace conditions.

To gain insight on the impact of saliva on aroma release depending on wine matrix composition, a LSD test was also carried out for each type of wine and for each aroma compound. **Table 3** shows these results taking into consideration the different aroma chemical families assayed. As it can be seen, in general, the addition of saliva (artificial or human) provoked a significant decrease (or higher retention) on the aroma release for most of the aroma compounds assayed. However, the extent of this effect was dependent on the type of wine, but also on the type of aroma chemical class. In this sense, it is important to highlight that human saliva exerted a high impact on the aroma release from red wines and practically all the aroma compounds assayed were less released when human saliva was added to the wine. However, in the case of white wines this effect was more dependent on the type of aroma compound. For example, the addition of human or artificial saliva did not affect the release of any of the alcohols of the aroma mixture. As it can be seen in the table, the effect of saliva seemed to be much lower in the case of synthetic wines.

Table 3. Average aroma release values and results of LSD test in the wines determined by static HS-SPME-GC-MS.

	Synthetic wine			White wine			Red wine		
	Water	Artificial saliva	Human saliva	Water	Artificial saliva	Human saliva	Water	Artificial saliva	Human saliva
Terpenes									
α-pinene	495.6 b	398.4 a	412.7 a	466.3 b	392.7 a	381.6 a	560.3 c	442.8 a	517.5 b
β-pinene	584.8 b	483.3 a	488.4 a	536.5 b	461.1 a	432.7 a	648.0 c	533.0 a	574.1 b
Limonene	95.1 b	76.5 a	77.9 a	89.0 b	72.8 a	72.1 a	99.5 b	82.9 a	82.5 a
Linalool	2.3 a	2.1 a	2.2 a	2.2 a	2.1 a	2.1 a	2.4 b	2.4 ab	2.2 a
Terpinen-4-ol	1.8 a	1.6 a	1.6 a	1.7 a	1.6 a	1.7 a	1.8 b	1.7 ab	1.6 a
α-terpineol	1.2 b	1.0 a	1.0 ab	1.2 b	1.1 a	1.2 b	1.2 b	1.0 a	1.0 a
β-citronellol	2.3 b	2.0 a	2.0 a	2.1 a	2.0 a	2.0 a	2.3 b	2.1 ab	2.0 a
Nerol (cis-geraniol)	1.7 b	1.5 a	1.5 ab	1.6 b	1.5 a	1.4 a	1.7 b	1.6 ab	1.5 a
Esters									
Ethyl propanoate	19.1 b	17.9 a	19.0 b	17.9 a	17.6 a	17.2 a	20.1 a	18.9 a	18.4 a
Isobutyl acetate	11.8 b	11.0 a	11.6 b	10.8 a	10.9 a	10.5 a	12.2 a	11.4 a	11.2 a
Ethyl butanoate	39.1 ab	38.2 a	41.4 b	35.9 a	36.1 a	38.0 a	41.9 b	38.1 a	36.9 a
Ethyl 2-methylbutanoate	29.4 c	26.6 a	27.7 b	26.4 a	25.5 a	25.4 a	30.4 b	27.9 ab	27.5 a
Butyl acetate	33.6 a	31.7 a	27.0 a	31.6 a	30.8 a	31.0 a	36.5 b	34.1 a	32.8 a
Isoamyl acetate	78.9 c	71.3 a	74.5 b	72.9 a	71.2 a	70.1 a	82.7 b	74.5 a	73.5 a
Ethyl hexanoate	194.5 b	173.2 a	179.0 a	179.9 b	169.7 a	170.6 a	201.4 b	183.7 a	176.6 a
Hexyl acetate	116.9 b	104.7 a	106.8 a	107.7 b	102.3 a	102.7 a	121.3 b	111.1 a	106.1 a
Ethyl octanoate	101.0 b	83.8 a	85.8 a	88.5 b	82.1 a	82.3 a	95.0 b	87.5 a	82.5 a
Ethyl decanoate	122.2 b	96.5 a	102.0 a	104.1 b	93.4 a	93.0 a	102.8 b	106.5 b	93.8 a
Diethyl succinate	1.6 a	1.4 a	1.5 a	2.2 b	2.0 a	2.1 ab	2.9 b	3.4 c	1.7 a
Beta-phenylethyl acetate	13.4 b	12.2 a	12.5 ab	12.8 a	12.4 a	12.6 a	13.6 b	12.7 ab	12.1 a
Ethyl dodecanoate	294.3 b	216.3 a	212.4 a	215.8 b	187.4 a	182.4 a	218.5 b	243.5 c	163.3 a
Alcohols									
Isobutanol	0.1 a	0.1 a	0.1 a	0.1 a	0.1 a	0.1 a	0.3 b	0.3 b	0.2 a

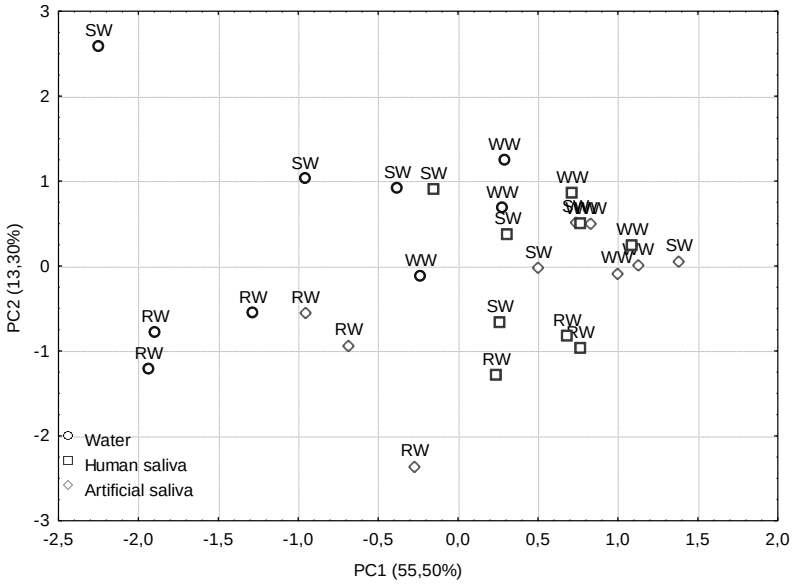
1-butanol	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a	1.2 c	1.1 b	1.0 a
Isoamylic alcohols	53.4 a	52.1 a	56.1 a	52.7 a	52.1 a	51.1 a	59.8 b	58.1 b	54.0 a
1-hexanol	8.4 a	8.1 a	8.2 a	8.1 a	8.1 a	8.0 a	9.2 b	8.6 a	8.2 a
trans-3-hexen-1-ol	1.0 a	0.9 a	1.0 a	1.0 a	0.9 a	1.0 a	1.1 b	1.0 ab	1.0 a
cis-3-hexen-1-ol	1.1 a	1.0 a	1.0 a	1.0 a	1.0 a	1.0 a	1.1 b	1.1 ab	1.0 a
Benzyl alcohol	0.4 a	0.3 a	0.4 a	0.4 a	0.3 a	0.4 a	0.4 a	0.4 a	0.4 a
β-phenylethyl alcohol	3.8 a	3.1 a	3.6 a	3.4 a	2.9 a	3.0 a	3.7 a	4.0 a	3.4 a
Lactones/Furanic									
Furfural	3.0 b	2.7 a	2.9 b	2.7 b	2.6 a	2.7 ab	3.0 b	2.8 b	2.7 a
5- methylfurfural	1.3 b	1.1 a	1.2 ab	1.2 a	1.1 a	1.2 a	1.3 b	1.2 b	1.2 a
γ-butyrolactone	0.2 a	0.2 a	0.2 a	0.3 b	0.2 a	0.2 ab	0.4 a	0.5 a	0.4 a
Furfuryl alcohol	0.2 a	0.2 a	0.2 a	0.2 a	0.2 a	0.2 a	0.2 a	0.2 a	0.2 a
trans-whiskey lactone	0.9 a	0.8 a	0.8 a	0.8 a	0.8 a	0.8 a	0.9 a	0.9 a	0.8 a
cis-whiskey lactone	0.7 a	0.6 a	0.6 a	0.6 a	0.6 a	0.6 a	0.7 b	0.6 ab	0.6 a
γ-nonalactone	0.5 a	0.4 a	0.4 a	0.4 a	0.3 a	0.4 a	0.4 b	0.4 b	0.3 a
Volatile phenols									
2-methoxy,4-ethylphenol	1.7 b	1.5 a	1.5 ab	1.6 a	1.5 a	1.6 a	1.7 b	1.6 b	1.5 a
Eugenol	0.3 b	0.3 a	0.3 ab	0.3 a	0.3 a	0.3 a	0.3 b	0.3 b	0.3 a
4-ethylphenol	1.3 a	1.1 a	1.2 a	1.2 a	1.1 a	1.1 a	1.3 a	1.3 a	1.2 a
C13-norisoprenoids									
β-damascenone	1.6 a	1.5 a	1.6 a	1.6 a	1.5 a	1.5 a	1.6 b	1.5 ab	1.4 a
α-ionone	2.5 a	2.3 a	2.3 a	2.4 b	2.2 a	2.3 a	2.4 b	2.3 b	2.2 a
β-ionone	4.4 b	4.0 a	3.9 a	4.2 b	3.9 a	4.0 ab	4.2 b	4.0 ab	3.7 a
Acids									
Hexanoic acid	1.0 b	0.8 a	1.0 b	0.9 a	0.8 a	0.9 a	1.1 b	0.8 a	0.9 a
Octanoic acid	2.7 b	2.2 a	2.7 ab	2.6 b	2.4 a	2.3 a	2.8 b	2.1 a	2.3 a
Decanoic acid	2.5 b	1.3 a	1.4 a	1.5 b	1.3 ab	1.2 a	1.4 b	1.1 a	1.4 b

All values (area: arbitrary unit) are divided by a factor of 10.000. Different letters for the same aroma compound in the same wine type (synthetic, white, red) denote statistical differences among saliva types after applying LSD test.

To better understand the way in which both factors (type of saliva and wine matrix) affected the aroma release behavior, a PCA was also performed taking into consideration all the aroma release data. Two principal components, PC1 and PC2 explaining 68.8 % of data variations were obtained (**Figure 2a**). As it can be seen in the graph, PC1 was mainly involved in the separation of the samples depending on the type of medium (with human saliva, artificial saliva or water). In agreement with previous results, the clearest separation (or differences) among wine samples were obtained for red wines. As it can be seen, red wines with human saliva showed positive values for PC1 while red wines with water showed high and negative values for this component. Red wines with artificial saliva showed an intermediate behavior and were placed between the other two types of wine samples (with human saliva and water). PC1 was negatively correlated with many volatile compounds (twenty five volatile compounds showed loadings lower than 0.8 and fifteen of them lower than 0.9). Among them, the variable projection (**Figure 2 b**) showed that some aroma compounds such as limonene (11), hexyl acetate (14), *cis*-3-hexen-1-ol (17), linalool (20) or 5-methylfurfural (21) among others, were strongly correlated with PC1. On the contrary, PC2 separated wines in function of wine type. Red wines exhibited negative values for this component, whilst white and synthetic wines appeared on the half top of the graph showing positive values for PC2.

These results underlined an effect of saliva on aroma release dependent on wine matrix composition. Even more interestingly, red wines seemed to be more affected than white and synthetic wines. The most outstanding effect provoked by human saliva was a reduction on the aroma release of most of the aroma compounds independently of their chemical structure. This global effect could be the result of the combination of single effects that could be differently affecting the volatile compounds employed in this study.

2a)



2b)

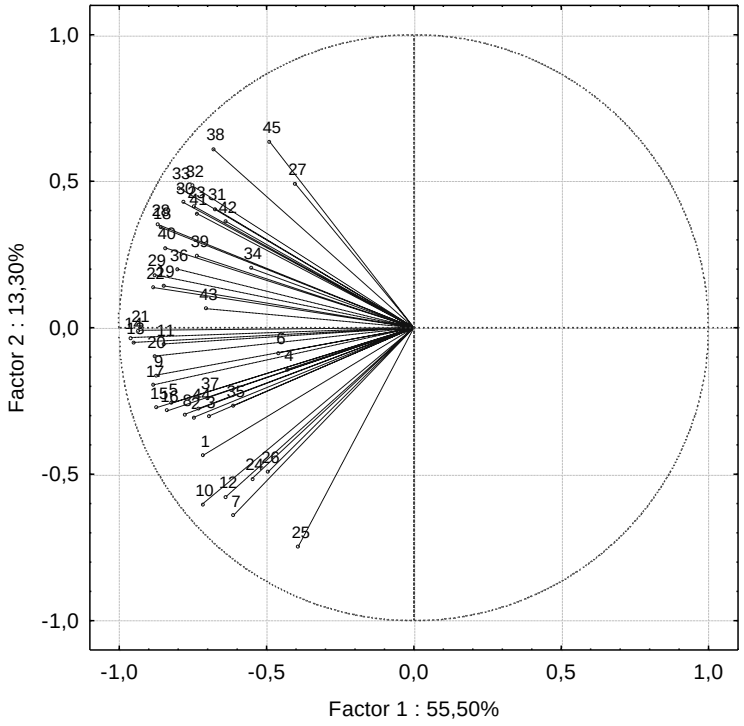


Figure 2. Graphic representation of the wine samples (2a) and of the variables (2b) obtained using PCA with the aroma release data from the static HS-SPME-GCMS. Numbers of the variables in Figure 2b correspond to the compounds listed in **Table 1.**

For instance, it is already known that wine polyphenols, which are more abundant and structurally different in red than in white wines, might interact with aroma compounds through different mechanisms depending on polyphenol structure decreasing the amount of aroma release (Dufour and Bayonove 1999; Jung et al. 2000; Aronson and Ebeler 2004; Rodriguez-Bencomo et al. 2011). In addition to this effect, wine polyphenols (such as procyanidins) might form insoluble complexes with saliva proteins with colloidal structures(Mitropoulou et al. 2011)modifying the viscosity of the sample, and therefore, affecting aroma release. To check this hypothesis, the viscosity values of white and red wines with the two types of saliva and water were also determined. **Table 4** shows that the viscosity values determined in all the wines were very similar ranging from 6.9 mPa*s for the white wine with water to 7.3 mPa*s for the white wine with artificial saliva. Therefore, there were not any substantial differences between red or white wines. Although an increase in viscosity induced by saliva has been proposed in order to explain the lower aroma release observe in oil/water emulsions (Buettner and Beauchamp 2010; Benjamin et al. 2012), the low volume of saliva compared to the wine (1:5) employed in this study, might not be enough to provoke a clear effect, at least in static headspace conditions as used here. Therefore, this factor did not seem a determinant parameter responsible for the higher retention of aroma compounds determined in red wines and specifically in those with human saliva.

The buffering capacity of saliva might be another important factor to explain aroma release, since this property might induce changes in the pH of the food matrix (Buettner and Beauchamp 2010; Salles et al. 2011). In fact, this factor has been pointed out, since it might influence the overal perception of aroma compounds during the *in vivo* consumption of palm-wine (Lasekan et al. 2009). To check this hypothesis, the pH values of the human and artificial saliva and the pH values of the wine/saliva mixtures were determined and they are also shown in **Table 4**. The original pH value for the artificial saliva was a little bit higher (8.4) than the pH of the human saliva (8.2). As expected and for both white and red wines, the addition of water practically did not change the pH while it increased with the addition of saliva. Artificial saliva seemed to induce higher changes in pH than human saliva and this could be due to its higher original pH compared to the human saliva. Therefore, differences induced by changes in pH did not seem relevant to explain the differences in the behavior of the aroma compounds in both wines whatever the matrix and the type of medium.

Table 4. Viscosity and pH values determined for the saliva samples and wine/saliva mixtures (*n*=3).

	Viscosity (mPa x s)			pH		
	Water	Human saliva	Artificial saliva	Water	Human saliva	Artificial saliva
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Salivas	-	7.0 (0.1)	7.9 (0.2)	-	8.2 (0.1)	8.4 (0.2)
Red wine	7.2 (0.1)	7.3 (0.1)	7.1 (0.1)	3.8 (0)	4.0 (0.1)	4.2 (0.1)
White wine	6.9 (0.0)	7.3 (0.3)	7.4 (0.5)	3.1 (0)	3.3 (0.1)	3.7 (0.1)

To explain the retention effect induced by saliva, mainly in red wines, we have to propose additional hypothesis. Previous works using static headspace conditions but with other food matrices have shown the ability of the saliva protein mucin to bind aroma compounds via hydrophobic interactions leading to a reduction in aroma release into the headspace (Friel and Taylor 2001). Moreover, this hypothesis has already been proposed to explain the lower release of a wide range of volatile compounds (e.g. esters, acetates, alcohols) from red and white wines (Genovese et al. 2009). However, in the present study, red wine with human saliva released lower amounts of aroma than the same wine with artificial saliva. The final amount of mucin in the wine/artificial saliva vial was 4.32 mg, while the amount of total protein (including mucin) in the wines samples added with human saliva was lower; 0.98 mg. Therefore, wines spiked with human saliva should have a minor interaction effect with mucin (and therefore higher aroma release) than wines spiked with artificial saliva, which does not explain our results. However, it is important to bear in mind, that human saliva contains other proteins different to mucin, for instance, proline-rich proteins (PRPs), histidine rich proteins (histatins or HRPs), lactoferrine, and enzymes (α-amylase, lipase, etc) (McRae and Kennedy 2011), which could be also involved in specific interactions with aroma compounds explaining the lower aroma release of wines with human saliva. In particular, PRPs, which represent up to 70 % of proteins originated from the parotid gland, are known to interact with tannins leading to the formation of some aggregates (Canon et al. 2013). Moreover, it has been shown that depending on the protein and tannin concentrations, dense aggregates might coexist with non-aggregated proteins, the latter also showing a significant number of bound tannin molecules (Canon et al. 2013). We could hypothesize that the formation of this second type of aggregates might

interact with aroma molecules without substantially changing the viscosity of the solution, as it was observed in the present study in the case of red wines added with human saliva.

Moreover, besides tannins, other wine matrix components might be also involved in the formation of these types of aggregates. Mitropoulou et al., (Mitropoulou et al. 2011) have suggested, at least in reconstituted model wines, the possible formation of saliva protein-polyphenol-carbohydrate complexes able to encapsulate hydrophobic aroma molecules. In this sense, in addition to the higher concentration of polyphenols determined in the red wine employed in this study, the polysaccharide content was also higher (2502 mg mannose/L) compared to the white wines (1667 mg mannose/L) (**see Table 2**). The formation of these type of complexes involving saliva proteins and specific wine polyphenols (tannins) and polysaccharides, both at higher concentrations in red than in white wines, might explain why red wines, and specifically those with human saliva retained more aroma molecules. Moreover, the fact that the very high hydrophobic aroma compounds (log *P*>2) of the aroma mixture showed higher retention (lower aroma release) in red wines with saliva than in the white wines, might be in agreement with this hypothesis. The formation of these structures (protein-polyphenol-carbohydrate complexes) might, however, represent a reservoir of aroma molecules ready to be released by the exhalation breath during the *in vivo* red wine consumption, as it has been recently proposed (Munoz-Gonzalez et al. 2014).

Finally, the salivary metabolic activity might have also affected the release of certain aroma compounds. In this regard, a reduction of aldehydes to the corresponding alcohols and/or partial hydrolysis of certain aroma compounds such as esters might be expected (Buettner 2002; Buettner 2002). In the case of the aldehydes employed in the aroma mixture (furfural and 5-methyl furfural), the release of these compounds was lower in red wines with human saliva, which could be in agreement with a possible transformation by an NADP-linked aldehyde reductase (Buettner 2002). However, the increase of the corresponding alcohol (furfuryl alcohol in the case of furfural) was not significant in these samples. In addition, the involvement of aldehydes in the formation of condensation products such as Schiff bases (e.g. with salivary proteins) or other chemical reactions might be also possible (Buettner 2002).

On the other hand, a reduction of esters in the HS of white but mainly red wines with saliva has been also shown in this work. However, this seems to be more related to the interaction of these compounds with the complex protein-polyphenol-carbohydrate than related to the esterase activity of saliva. Although the decrease in the release of some esters (ethyl butanoate, hexanoate, octanoate, etc) in wines with human saliva compared to control wines (without saliva) has been attributed to the esterase activity (Genovese et al. 2009), the activity of these enzymes has only been proven in a very different environment (specifically, in an aqueous system at pH 5) (Buettner 2002) to that accounted for in wine (12% ethanolic system at pH 3.5). Therefore, it seems difficult to obtain straightforward relationships between the decrease in ester release and saliva esterase activity.

Effect of saliva on aroma release using dynamic headspace conditions

In the present work, aroma release from different wine matrices in dynamic conditions was determined by using a bioreactor cell with controlled temperature and agitation conditions at two different sampling times (initial t=0 min and final t=10 min) (**Figure 1**). The initial sampling time (t=0) might be related to the oral phase, in which the mixture of wine (generally cold) and saliva is at lower temperature (25.5 ºC) than physiological temperature (36 ºC). The final sampling time (t=10) could be more representative of the post-oral phase, in which some volatiles could be released from the liquid sample remaining in the oral cavity after drinking (Buettner et al. 2001) at oral temperature (36 ºC).

Aroma release data collected from t=0 and t=10 minutes are shown in **Tables 5** and **6** respectively. These data were submitted to two independent two way ANOVA (one for each sampling time), considering the global effect of saliva type (artificial, human, water) and type of wine matrix (red, white, synthetic). Results showed that in the oral-phase (t=0) only nine aroma compounds were affected by saliva type while thirty of them were affected by wine matrix (data not shown). In addition, eleven compounds showed an effect of the interaction factor. In the case of the post-oral phase (t=10 minutes) the application of the same statistical treatment also showed a higher influence of wine matrix composition (22 compounds significantly affected) compared to the saliva effect (7 compounds) and the interaction (5 compounds) (data not shown).

Compared to results from the previous static headspace analysis the influence of saliva on aroma release seemed to be lower. The minor effect of saliva addition by using the dynamic approach compared to the static headspace analysis could have been due to a displacement of the equilibrium, which might reduce the retention effect produced by proteins(Fabre et al. 2002) or by other non-volatile wine matrix molecules, such as the above mentioned protein-polyphenol-polysaccharides complexes. These findings are not surprising taking into consideration that several authors have already suggested that in spite that dynamic conditions might better simulate the consumption situation, static measurements are better suited for determining thermodynamic and kinetic parameters with good precision (Juteau et al. 2004).

In terms of amount of aroma release, it is interesting to notice that higher release for most of the aroma compounds were found during the post-oral release step (t=10 minutes) (**Table 6**) compared to the oral phase (**Table 5**). This could be due to the higher extraction temperature in the post-oral phase (36 ºC) compared to the oral phase (25.5 ºC). Previously, the effect of temperature (4, 23 and 60 ºC) on volatile release from oil/water emulsions using an artificial mouth system had been pointed out (Benjamin et al. 2012). These authors showed a similar effect between 4 and 23 ºC (release less pronounced), compared to 60 ºC. In the present work, using more realistic temperatures closer to what was expected during wine consumption (25.5 ºC and 36 ºC), most of the volatile compounds showed higher release when the temperature raised about 12 ºC independent of the wine type. The increase in sampling temperature increases the partitioning of the volatiles into the gas phase following the vant'Hoff 's law (Tromelin et al. 2010). In addition, in ethanol solutions (as wine) and using dynamic headspace conditions, Tsachaki et al. (2008) showed that the evaporation of ethanol at the solution vapor interface might create a surface tension gradient, making new ethanol molecules move from the bulk phase to replenish the depleted surface areas, carrying along an appreciable volume of underlying liquid with aroma compounds. This phenomenon, called the Marangoni effect (Spedding et al. 1993), might also explain the higher aroma release for most of the volatile compounds in the wines with a moderate increase in temperature.

To extract more conclusions on the role of saliva on aroma release using dynamic conditions, a LSD test for mean comparison was also performed for each type of wine (red, white and artificial) in the oral and post-oral phases. These results are also

shown in **Tables 5** and **6**. Results show that during the oral-phase (t=0)**,** only three terpenes (α- and β-pinene and limonene) showed significant lower release in the three types of wines with saliva (human and artificial) (**Table 5**). The same compounds were significantly less released in white and red wines with saliva during the post-oral phase (t=10) (**Table 6**). These compounds are characterized by high log *P* values, which seem to be in agreement with their involvement in the formation of hydrophobic interactions with wine polyphenols (Dufour and Bayonove 1999) or in their involvement in the formation of complexes with salivary proteins, polyphenols and polysaccharides.

Surprisingly and mainly during the oral-phase, a relatively high number of aroma compounds were highly released in the wines with saliva, which seem to contradict results from the previous experiment performed in static conditions. This could be due to the higher sensitivity of the dynamic HS conditions over the static HS, which might have improved the detection of some aroma compounds (Fabre et al. 2002). For instance, some lactones (*cis*- and *trans*- whiskylactones, γ-nonalactone), furanic compounds (furfural, 5-methylfurfural), volatile phenols (eugenol, ethylphenol), C13 norisoprenoids (β-damascenone, α-ionone, β-ionone), and terpene alcohols (linalool, terpinen-4-ol, α-terpineol, β-citronellol, nerol) were more highly released in red wines with human saliva (**Table 5**).

Some of these compounds such as terpene alcohols, could have originated "de novo" from the corresponding grape glycosidic precursor. These non-volatile compounds could have remained in the non-volatile wine matrix after the dearomatisation step in higher amounts in the red than in the white wine. The higher release of some ethyl phenols in red wines in the presence of human saliva could be explained by the presence of a cinnamate reductase activity acting directly on red wine vinyl phenols. These compounds may also be generated after a previous decarboxilation step of red wine phenolic acids, also more abundant in red than white wines. However, these hypotheses need to be confirmed in further experiments, since it is also certain that previous studies have indicated the role of some polyphenols such as tannins on the inhibition of certain enzymes (such as glycosidases) (Vasserot et al. 1993; Juntheikki and Julkunen-Tiito 2000).

Table 5. Average aroma release values and results of LSD test in the wines determined by dynamic HS-SPME-GC-MS at t=0 (oral-phase).

	Synthetic wine			White wine			Red wine		
	Water	Artificial saliva	Human saliva	Water	Artificial saliva	Human saliva	Water	Artificial saliva	Human saliva
Terpenes									
α-pinene	49.1 b	0.2 a	19.0 a	7.2 b	2.0 a	0.7 a	10.9 c	1.3 a	3.7 b
β-pinene	45.1 b	0.0 a	16.3 a	9.9 b	3.2 a	1.1 a	19.4 c	3.3 a	7.3 b
Limonene	14.8 b	0.9 a	7.1 a	4.6 b	3.4 b	1.6 a	3.9 c	1.4 a	2.3 b
Linalool	1.7 a	1.6 a	1.7 a	1.9 a	1.9 a	1.9 a	1.8 a	1.6 a	2.1 b
Terpinen-4-ol	1.2 a	1.1 a	1.1 a	1.3 a	1.3 a	1.3 a	1.3 ab	1.1 a	1.5 b
α-terpineol	1.0 a	1.1 a	0.9 a	0.7 a	0.8 a	0.8 a	0.7 a	0.6 a	0.9 b
β-citronellol	1.4 a	1.1 a	1.1 a	1.3 a	1.4 a	1.3 a	1.3 b	1.1 a	1.6 c
Nerol (cis-geraniol)	0.9 b	0.7 a	0.8 ab	0.9 a	0.9 a	0.9 a	0.9 ab	0.8 a	1.1 b
Esters									
Ethyl propanoate	37.8 a	36.1 a	39.7 b	39.1 a	41.8 a	41.2 a	38.6	37.1 a	36.8 a
Isobutyl acetate	19.2 b	16.8 a	18.9 b	19.0 a	20.0 a	19.2 a	18.5 c	17.2 a	17.8 b
Ethyl butanoate	58.9 b	52.0 a	60.6 b	58.5 a	63.0 a	58.2 a	57.5 c	53.5 a	55.5 b
Ethyl 2-methylbutanoate	34.8 b	28.7 a	32.1 ab	32.3 a	34.4 a	32.0 a	31.3 c	28.8 a	30.2 b
Butyl acetate	48.0 a	45.9 a	48.4 a	48.6 a	51.6 a	50.3 a	47.8 a	43.6 a	47.6 a
Isoamyl acetate	100.1 b	89.2 a	96.6 ab	94.7 a	104.4 a	88.8 a	94.1 a	82.9 a	94.1 a
Ethyl hexanoate	184.0 b	150.7 a	164.7 ab	169.9	176.4 a	164.3 a	151.0	136.4 a	153.7 a
Hexyl acetate	112.4 b	93.1 a	101.1 ab	104.7	108.3 a	101.7 a	91.8 a	82.6 a	93.6 a
Ethyl octanoate	65.9 b	46.6 a	50.1 a	56.5 a	59.7 a	53.3 a	42.2	38.2 a	43.5 b
Ethyl decanoate	35.4 b	19.5 a	19.3 a	25.0 a	26.4 a	22.2 a	17.8 a	15.3 a	17.8 a
Diethyl succinate	1.0 a	0.9 a	0.9 a	1.1 a	1.2 a	1.1 a	1.8 a	1.9 a	2.5 b
Beta-phenylethyl acetate	11.5 a	11.6 a	11.0 a	13.3 a	13.9 a	13.7 a	12.7	11.8 a	14.9 b
Ethyl dodecanoate	29.2 a	14.0 a	11.8 a	13.0 a	14.3 a	11.2 a	9.9 c	6.1 a	7.9 b
Alcohols									
Isobutanol	0.2 a	0.3 a	0.4 a	0.3 a	0.3 a	0.3 a	0.5 a	0.5 a	0.5 a
1-butanol	1.6 a	1.6 a	2.0 a	1.7 a	1.7 a	1.7 a	1.8 a	1.7 a	1.9 a
Isoamylic alcohols	68.0 a	77.3 b	75.9 b	78.7 a	80.7 a	79.6 a	83.0 a	78.4 a	85.3 a

1-hexanol	9.2 a	9.9 a	9.9 a	10.4 a	10.6 a	10.7 a	10.7 a	9.7 a	11.3 a
trans-3-hexen-1-ol	1.1 a	1.1 a	1.2 a	1.1 a	1.2 a	1.2 a	1.2 ab	1.1 a	1.3 b
cis-3-hexen-1-ol	0.9 a	0.9 a	1.1 b	1.0 a	1.0 a	0.9 a	1.0 ab	0.9 a	1.2 b
Benzyl alcohol	0.2 a	0.2 a	0.3 a	0.3 a	0.3 a	0.3 a	0.3 a	0.3 a	0.4 b
β-phenylethyl alcohol	1.9 a	1.9 a	1.9 a	2.1 a	2.6 a	2.5 a	2.1 a	2.3 a	3.2 b
Lactones/Furanic									
Furfural	2.9 a	2.7 a	3.2 b	2.1 a	2.3 a	2.1 a	2.7 a	2.6 a	3.3 b
5- methylfurfural	1.2 a	1.1 a	1.2 b	1.1 a	1.2 a	1.2 a	1.1 a	1.1 a	1.4 b
γ-butyrolactone	0.3 a	0.3 a	0.3 a	0.3 a	0.4 a	0.4 a	0.5 a	0.4 a	0.6 a
Furfuryl alcohol	0.1 a	0.1 a	0.1 b	0.1 a	0.2 a	0.2 a	0.1 a	0.1 a	0.2 a
trans-whiskey lactone	0.6 a	0.5 a	0.5 a	0.6 a	0.7 a	0.6 a	0.6 a	0.6 a	0.8 b
cis-whiskey lactone	0.4 a	0.4 a	0.3 a	0.4 a	0.5 a	0.4 a	0.4 a	0.4 a	0.6 b
γ-nonalactone	0.2 a	0.2 a	0.2 a	0.2 a	0.3 b	0.3 b	0.3 a	0.2 a	0.3 b
Volatile phenols									
2-methoxy,4-ethylphenol	1.0 a	1.0 a	0.9 a	1.1 a	1.2 a	1.2 a	1.1 a	1.1 a	1.4 a
Eugenol	0.1 a	0.2 a	0.1 a	0.2 a	0.2 b	0.2 b	0.1 a	0.2 ab	0.2 b
4-ethylphenol	0.7 a	0.7 a	0.6 a	0.8 a	0.9 a	0.9 a	0.7 a	0.8 a	1.1 b
C13-norisoprenoids									
β-damascenone	1.2 a	1.2 a	1.1 a	1.4 a	1.4 a	1.4	1.3 ab	1.1 a	1.5 b
α-ionone	1.9 a	1.6 a	1.5 a	2.0 a	2.0 a	1.9	1.8 ab	1.5 a	2.1 b
β-ionone	2.9 b	2.5 ab	2.2 a	3.1 a	3.1 a	3.1	2.8 a	2.5 a	3.4 b
Acids									
Hexanoic acid	0.6 b	0.5 a	0.7 b	0.8 a	0.8 a	0.7	0.3 a	0.5 a	0.9 a
Octanoic acid	1.2 a	1.1 a	1.1 a	1.6 a	2.2 a	1.5	1.8 a	1.5 a	2.0 a
Decanoic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd

All values (area: arbitrary unit) are divided by a factor of 10.000. Different letters (a-c) for the same aroma compound in the same wine type (synthetic, white, red) denote statistical differences among saliva types after applying LSD test

Table 6. Average aroma release values and LSD test in the wines determined by dynamic HS-SPME-GC-MS at t=10 (post oral-phase).

	Synthetic wine			White wine			Red wine		
	Water	Artificial saliva	Human saliva	Water	Artificial saliva	Human saliva	Water	Artificial saliva	Human saliva
Terpenes									
α-pinene	20.6 a	0.2 a	19.0 a	6.8 b	1.6 a	0.7 a	8.8 b	1.2 a	3.7 a
β-pinene	21.6 a	0.1 a	18.4 a	9.8 b	2.8 a	1.1 a	17.5 b	3.6 a	7.5 a
Limonene	11.9	1.3 a	9.7 a	5.5 c	3.6 b	2.2 a	4.2 c	1.7 a	2.7 b
Linalool	4.4 a	4.9 a	4.6 a	4.5 a	4.5 a	5.0 b	4.3 a	4.1 a	4.3 a
Terpinen-4-ol	3.6 a	3.8 a	3.4 a	3.5 a	3.5 a	3.6 a	3.4 a	3.2 a	3.3 a
α-terpineol	3.1 a	3.5 a	2.9 a	2.0 a	2.1 a	2.3 a	1.9 a	1.8 a	1.8 a
β-citronellol	4.1 a	4.0 a	3.7 a	3.8 ab	3.5 a	4.0 b	3.6 a	3.4 a	3.5 a
Nerol (cis-geraniol)	2.3 a	2.3 a	2.4 a	2.1 ab	2.0 a	2.4 b	2.2 a	2.1 a	2.2 a
Esters									
Ethyl propanoate	28.7 a	32.5 b	31.1 a	32.1 a	32.3 a	32.3 a	30.4 a	28.9 a	30.2 a
Isobutyl acetate	17.6 a	19.1 a	18.5 a	18.5 a	19.5 b	18.6 a	17.7 a	17.0 a	17.3 a
Ethyl butanoate	61.8 a	65.2 a	66.6 a	64.2 a	66.3 a	62.7 a	60.0 a	56.2 a	59.2 a
Ethyl 2-methylbutanoate	35.0 a	37.9 a	36.4 a	35.4 ab	36.9 b	34.8 a	33.5 b	31.9 a	32.2 ab
Butyl acetate	52.3 a	59.7 b	55.8 a	55.4 a	58.3 b	56.3 a	54.1 a	52.8 a	52.9 a
Isoamyl acetate	117.5	124.9 a	120.2 a	118.1 a	123.0 a	117.9 a	110.5 a	110.6 a	108.4 a
Ethyl hexanoate	256.6	264.6 a	257.3 a	242.0 a	248.2 a	240.6 a	216.9 a	206.5 a	210.6 a
Hexyl acetate	159.9	163.8 a	159.2 a	150.4 a	153.4 a	149.5 a	133.3 a	126.1 a	128.8 a
Ethyl octanoate	100.9	94.7 a	94.2 a	84.6 a	83.5 a	83.2 a	63.9 a	59.5 a	60.7 a
Ethyl decanoate	57.2 a	45.8 a	45.2 a	45.0 a	42.1 a	42.6 a	31.4 a	28.2 a	28.7 a
Diethyl succinate	2.2 a	2.1 a	2.1 a	2.3 a	2.2 a	2.4 a	4.2 a	3.7 a	4.0 a
Beta-phenylethyl acetate	25.6 a	29.5 a	26.7 a	28.3 a	26.6 a	28.8 a	26.9 a	25.5 a	25.8 a
Ethyl dodecanoate	50.5 a	31.7 a	30.3 a	25.6 b	21.0 a	20.3 a	17.1 b	10.7 a	12.8 a
Alcohols									
Isobutanol	0.2 a	0.3 a	0.3 a	0.3 a	0.3 a	0.3 a	0.5 a	0.5 b	0.5 a
1-butanol	1.9 a	2.4 a	2.4 a	2.4 a	2.5 a	2.4 a	2.3 a	2.4 a	2.3 a
Isoamylic alcohols	92.4 a	116.7 b	102.6 a	113.3 a	118.7 a	117.6 a	112.4 a	117.0 b	112.3 a

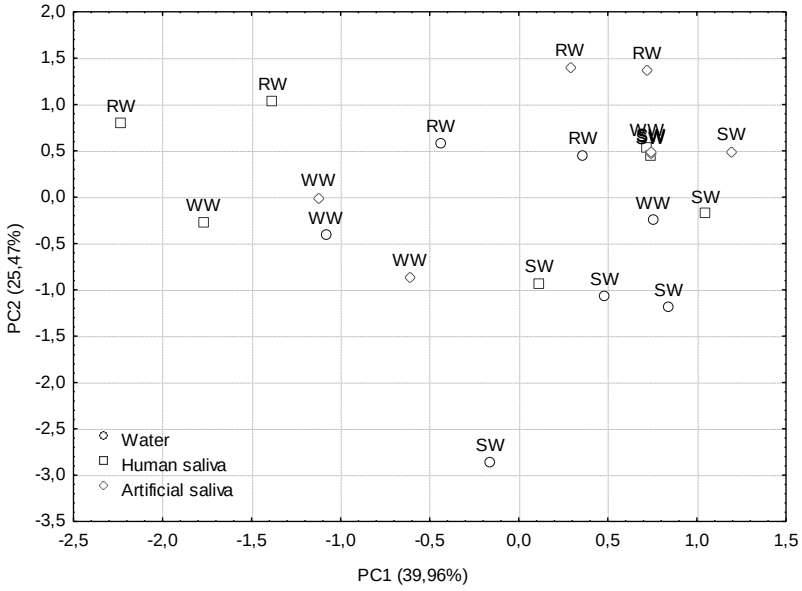
1-hexanol	17.5 a	21.2 b	19.0 a	19.6 a	20.3 a	20.3 a	19.5 a	19.1 a	19.2 a
trans-3-hexen-1-ol	2.1 a	2.5 a	2.4 a	2.3 a	2.9 a	2.5 a	2.3 a	2.2 a	2.2 a
cis-3-hexen-1-ol	1.7 a	2.1 b	2.1 b	2.1 a	2.1 a	2.3 a	2.0 a	2.0 a	2.2 b
Benzyl alcohol	0.4 a	0.4 a	0.4 a	0.4 a	0.4 a	0.4 a	0.4 a	0.4 a	0.4 a
β-phenylethyl alcohol	3.8 a	3.8 a	3.7 a	3.5 a	3.4 a	3.9 a	3.9 a	3.2 a	3.7 a
Lactones/Furanic									
Furfural	5.2 a	6.1 b	6.2 b	4.8 b	5.1 c	4.5 a	5.4 a	5.6 a	5.8 a
5- methylfurfural	2.2 a	2.4 a	2.5 a	2.4 a	2.3 a	2.4 a	2.4 a	2.2 a	2.5 a
γ-butyrolactone	0.4 a	0.5 a	0.3 a	0.4 a	0.5 a	0.5 a	0.6 a	0.5 a	0.6 a
Furfuryl alcohol	0.2 a	0.2 a	0.2 a	0.2 a	0.2 a	0.2 a	0.2 a	0.2 a	0.2 a
trans-whiskey lactone	1.4 a	1.4 a	1.3 a	1.3 a	1.3 a	1.4 a	1.4 a	1.3 a	1.3 a
cis-whiskey lactone	0.9 a	0.9 a	0.8 a	0.9 a	0.9 a	0.9 a	1.0 a	0.9 a	0.9 a
γ-nonalactone	0.5 a	0.5 a	0.5 a	0.5 b	0.5 a	0.5 b	0.5 a	0.4 a	0.5 a
Volatile phenols									
2-methoxy,4-ethylphenol	2.4 a	2.4 a	2.3 a	2.4 a	2.2 a	2.5 a	2.3 a	2.2 a	2.3 a
Eugenol	0.3 a	0.3 a	0.3 a	0.3 ab	0.3 a	0.4 b	0.3 a	0.3 a	0.3 a
4-ethylphenol	1.5 a	1.6 a	1.5 a	1.5 a	1.4 a	1.7 a	1.6 a	1.4 a	1.4 a
C13-norisoprenoids									
β-damascenone	3.4 a	3.8 a	3.4 a	3.6 a	3.6 a	3.8 a	3.3 a	3.1 a	3.2 a
α-ionone	5.4 a	5.5 a	4.9 a	5.2 a	5.2 a	5.5 a	4.7 a	4.3 a	4.5 a
β-ionone	7.8 a	8.1 a	7.3 a	8.3 a	8.0 a	8.7 a	7.4 a	6.7 a	6.9 a
Acids									
Hexanoic acid	1.0 a	0.9 a	1.1 a	1.4 a	1.4 a	1.6 b	1.3 b	1.0 a	1.1 a
Octanoic acid	2.5 a	2.1 a	2.6 a	3.6 b	3.1 a	4.0 b	3.0 a	2.5 a	2.4 a
Decanoic acid	nd	nd	nd	nd	nd	nd	nd	nd	nd

All values (area: arbitrary unit) are divided by a factor of 10.000. Different letters (a-c) for the same aroma compound in the same wine type (synthetic, white, red) denote statistical differences among saliva types after applying LSD test

In order to better understand the impact of saliva on aroma release in the three types of wine matrices, aroma release data (peak area) taken at t=0 and t=10 minutes were independently submitted to PCA. **Figure 3a** shows the representation of the two first principal components obtained after the application of this test to aroma release data collected from the wines at t=0. Both PCs explained more than 65% of data variation. As it can be seen(Figure 3), similarly to what happens in static conditions, the main differences among wine samples were found in the case of red wines. PC1 clearly separated among red wines with water and artificial saliva from red wines with human saliva. The latter exhibited high and negative values for this component. The representation of the variables on the basis of the two first components (**Figure 3b**) shows how some variables such as *trans* and *cis*-hexenol (16, 17), linalool (20), 5-methylfurfural (21), terpinen-4-ol (22), nerol (29), β-phenylethyl acetate (30), β-damascenone (32) and β-ionone (38) among others, were strongly and negatively correlated with PC1. Most of these compounds match with those previously shown in the LSD test (**Table 5**), as significantly more released in red wines with saliva, which is the same conclusion obtained by PCA. As said before, many of them could be the result of the enzymatic activity of saliva on certain aroma precursors that could have remained in higher amounts in the red wine matrix.

In **figure 3a**, PC2 also shows a separation of the samples depending on wine matrix composition. Red wines appeared on the top of the graph showing high values for this component, whilst white and mainly synthetic wines appeared in the low part of the graph with lower and even negative values for this component, especially in the water medium. As it can be seen (**Figure 3b**), the most correlated (negatively) variables were -in general, ethyl esters such as ethyl octanoate (18), ethyl decanoate (23) and ethyl dodecanoate (31) and and some nonalcoholic terpenes such α-pinene (3), β-pinene (8) and limonene (11). It seems that these compounds (with high Log P value) might interact more with saliva protein and wine matrix than with synthetic wine.

3a)



3b)

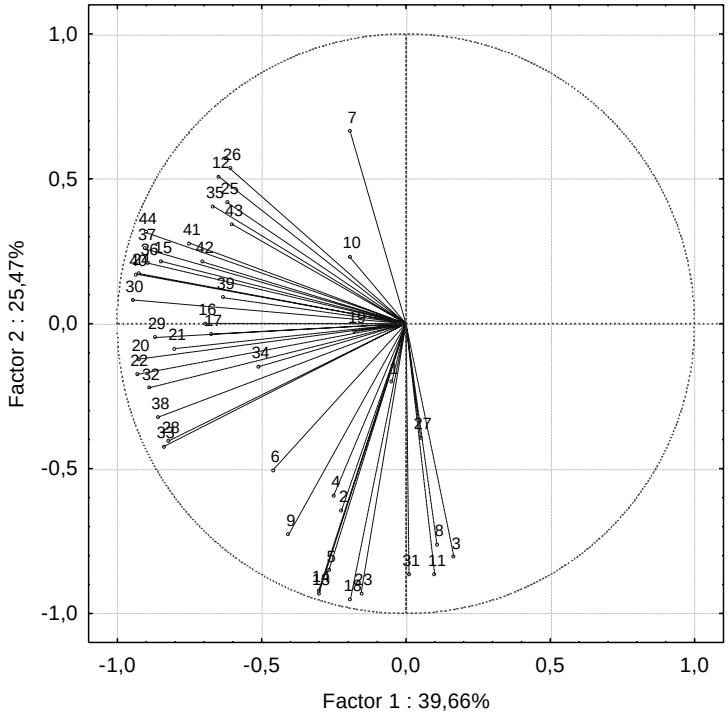
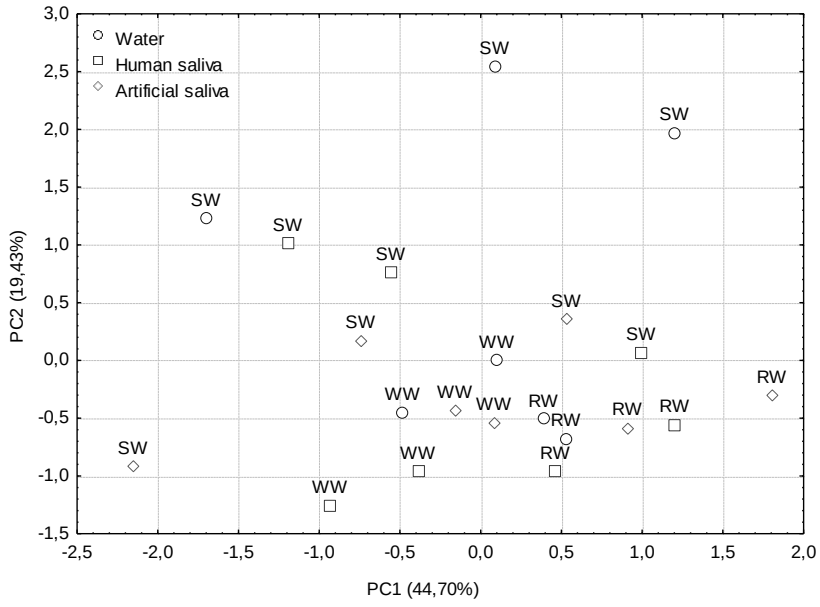


Figure 3. Graphic representation of the wine samples (2a) and of the variables (2b) obtained using PCA with the aroma release data from the dynamic HS-SPME-GCMS analysis at t=0 (oral phase). Numbers of the variables in Figure 3b correspond to the compounds listed in **Table 1**.

Similarly to the results found in the previous ANOVA and LSD test, results from the PCA performed with release data collected during the post-oral phase (t=10 minutes) did not show a clear grouping of wine samples depending on the medium composition (with human saliva, artificial saliva or water) (**Figure 4a**). However, an influence on the wine matrix composition was indeed manifested. As it can be seen, PC1 separates between red on the positive side of the graph and white and synthetic wines on the other side (**Figure 4a**) showing differences on their aroma release behavior. Red wines exhibited higher values for this component than white wines. The projection of the variables on the plane defined by the first and second components (**Figure 4b**) shows that PC1 was highly correlated (negatively) with some aroma compounds such as, terpinen-4-ol (22), β-citronellol (28), α-ionone (33), β-phenylethyl alcohol (37), 4-ethylguaicol (40) and 4-ethyl phenol (44), among others. In addition, PC2 also allowed a separation between synthetic wines with positive values for this component and white and red wines with negative values for it. In this case, PC2 was strongly and negatively correlated with some alcohols such as 1-butanol (10) or isoamylic alcohols (12) but positively with some non-alcoholic terpenes such as α- and β- pinene (3, 8) and limonene (11).

4a)



4b)

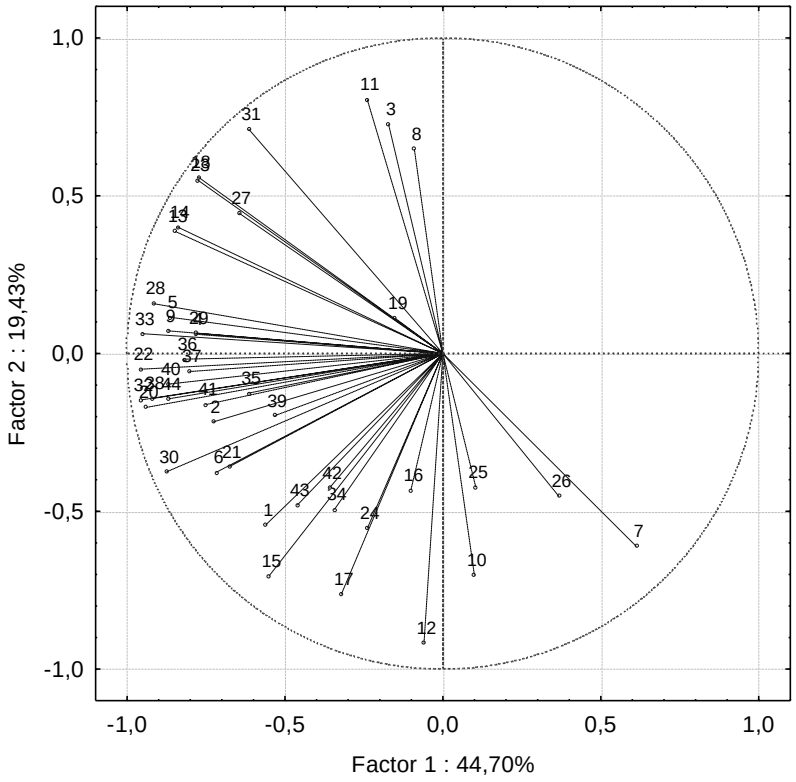


Figure 4. PCA of the Graphic representation of the wine samples (2a) and of the variables (2b) obtained using PCA with the aroma release data from the dynamic HS-SPME-GCMS analysis at t=10 (post oral phase). Numbers of the variables in Figure 4b correspond to the compounds listed in **Table 1**.

In conclusion, the main finding of this work is that saliva has an important effect on aroma release from wine and this effect was different depending on wine matrix composition. In addition, we found differences depending on using human or artificial saliva, therefore proving that other proteins than mucins seem to have an important role on aroma release. Moreover, it has been shown that the effect of saliva on wine aroma release is more evident when using static than dynamic headspace conditions. In general, human saliva produces lower release for most of the wine volatile compounds, and this effect was more important in red than white wines. The interaction of aroma compounds with other proteins different to mucin and/or the formation of complexes involving saliva glycoproteins-wine polyphenols-wine polysaccharides and aroma compounds, preferentially for those aroma compounds with high log *P* values (hydrophobic), seem to be responsible for the observed effect. However, the enzymatic

activity of saliva (human saliva) could also be involved in the higher aroma release observed for some specific compounds (mainly those from glycosidic aroma precursors) in red wines when using the more sensitive dynamic headspace approach. In addition, large differences in the amount of aroma released depending on sampling temperature during the oral and post-oral phases invite us to think about the importance of this second step of wine consumption as a mechanism in releasing aroma compounds from oral or throat wine depots influencing long lasting perception of aroma after swallowing. Finally, in spite of the minor impact of saliva observed in dynamic conditions, it is important to bear in mind that *in vivo* consumption conditions, could represent a more dynamic process to that used in the present work, in which saliva is continuously produced and replenished (incorporating more proteins to interact with aroma compounds, or enzymes) and also "fresh" sample is continuously being provided. Therefore, the extent of its effect could be higher than that determined with the experimental *in vitro* dynamic headspace conditions used in this study.

References

Ai J (1997). Solid phase microextraction for quantitative analysis in nonequilibrium situations. Analytical Chemistry 69(6): 1230-1236

Aronson J, Ebeler SE (2004). Effect of Polyphenol compounds on the headspace volatility of flavors. American Journal of Enology and Viticulture 55(1): 13-21

Benjamin O, Silcock P, Beauchamp J, Buettner A, Everett DW (2012). Tongue Pressure and Oral Conditions Affect Volatile Release from Liquid Systems in a Model Mouth. Journal of Agricultural and Food Chemistry 60(39): 9918-9927

Boland AB, Buhr K, Giannouli P, van Ruth SM (2004). Influence of gelatin, starch, pectin and artificial saliva on the release of 11 flavour compounds from model gel systems. Food Chemistry 86(3): 401-411

Buettner A (2002). Influence of human saliva on odorant concentrations. 2. aldehydes, alcohols, 3-alkyl-2-methoxypyrazines, methoxyphenols, and 3-hydroxy-4,5-dimethyl-2(5H)-furanone. Journal of Agricultural and Food Chemistry 50(24): 7105-7110

Buettner A (2002). Influence of human salivary enzymes on odorant concentration changes occurring in vivo. 1. Esters and thiols. Journal of Agricultural and Food Chemistry 50(11): 3283-3289

Buettner A, Beauchamp J (2010). Chemical input - Sensory output: Diverse modes of physiology-flavour interaction. Food Quality and Preference 21(8): 915-924

Buettner A, Beer A, Hannig C, Settles M (2001). Observation of the swallowing process by application of videofluoroscopy and real-time magnetic resonance imaging-consequences for retronasal aroma stimulation. Chemical Senses 26(9): 1211-1219

Buettner A, Beer A, Hannig C, Settles M, Schieberle P (2002). Physiological and analytical studies on flavor perception dynamics as induced by the eating and swallowing process. Food Quality and Preference 13(7-8): 497-504

Buettner A, Schieberle P (2000). Exhaled odorant measurement (EXOM) - A new approach to quantify the degree of in-mouth release of food aroma compounds. Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology 33(8): 553-559

Burdach KJ, Doty RL (1987). The effects of tongue movements and swallowing on retronasal aroma perception. chemical senses 12(1): 181-181

burdach kj, kroeze jha, koster ep (1984). Nasal, retronasal, and gustatory perception - an experimental comparison. perception & psychophysics 36(3): 205-208

Cala O, Dufourc EJ, Fouquet E, Manigand C, Laguerre M, Pianet I (2012). The Colloidal State of Tannins Impacts the Nature of Their Interaction with Proteins: The Case of Salivary Proline-Rich Protein/Procyanidins Binding. Langmuir 28(50): 17410-17418

Canon F, Pate F, Cheynier V, Sarni-Manchado P, Giuliani A, Perez J, Durand D, Li J, Cabane B (2013). Aggregation of the Salivary Proline-Rich Protein IB5 in the Presence of the Tannin EgCG. Langmuir 29(6): 1926-1937

Conner JM, Birkmyre L, Paterson A, Piggott JR (1998). Headspace concentrations of ethyl esters at different alcoholic strengths. Journal of the Science of Food and Agriculture 77(1): 121-126

de Freitas V, Mateus N (2012). Protein/Polyphenol Interactions: Past and Present Contributions. Mechanisms of Astringency Perception. Current Organic Chemistry 16(6): 724-746

Drobitch RK, Svensson CK (1992). Therapeutic drug-monitoring in saliva - an update. clinical Pharmacokinetics 23(5): 365-379

Dufour C, Bayonove C (1999). Interactions between Wine Polyphenols and Aroma Substances. An Insight at the Molecular Level. Journal of Agricultural and Food Chemistry 47(2): 678-684

Escalona H, Piggott JR, Conner JM, Paterson A (1999). Effect of ethanol strength on the volatility of higher alcohols and aldehydes. Italian Journal of Food Science 11(3): 241-248

Escudero A, Campo E, Farina L, Cacho J, Ferreira V (2007). Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. Journal of Agricultural and Food Chemistry 55(11): 4501-4510

Fabre M, Aubry V, Guichard E (2002). Comparison of different methods: Static and dynamic headspace and solid-phase microextraction for the measurement of interactions between milk proteins and flavor compounds with an application to emulsions. Journal of Agricultural and Food Chemistry 50(6): 1497-1501

Ferreira V, Ortin N, Escudero A, Lopez R, Cacho J (2002). Chemical characterization of the aroma of Grenache rose wines: Aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. Journal of Agricultural and Food Chemistry 50(14): 4048-4054

Friel EN, Taylor AJ (2001). Effect of salivary components on volatile partitioning from solutions. Journal of Agricultural and Food Chemistry 49(8): 3898-3905

Genovese A, Piombino P, Gambuti A, Moio L (2009). Simulation of retronasal aroma of white and red wine in a model mouth system. Investigating the influence of saliva on volatile compound concentrations. Food Chemistry 114(1): 100-107

Guth H (1997). Identification of character impact odorants of different white wine varieties. Journal of Agricultural and Food Chemistry 45(8): 3022-3026

Hansson A, Giannouli P, Van Ruth S (2003). The influence of gel strength on aroma release from pectin gels in a model mouth and in vivo, monitored with proton-transfer-reaction mass spectrometry. Journal of Agricultural and Food Chemistry 51(16): 4732-4740

Harrison M (1998). Effect of breathing and saliva flow on flavor release from liquid foods. Journal of Agricultural and Food Chemistry 46(7): 2727-2735

Hussein MM, Kachikian R, Pidel AR (1983). Analysis for flavor residuals in the mouth by gas-chromatography. journal of food science 48(6): 1884-1885

Jung DM, de Ropp JS, Ebeler SE (2000). Study of interactions between food phenolics and aromatic flavors using one- and two-dimensional H-1 NMR spectroscopy. Journal of Agricultural and Food Chemistry 48(2): 407-412

Juntheikki MR, Julkunen-Tiito R (2000). Inhibition of beta-glucosidase and esterase by tannins from Betula, Salix, and Pinus species. Journal of Chemical Ecology 26(5): 1151-1165

Juteau A, Cayot N, Chabanet C, Doublier JL, Guichard E (2004). Flavour release from polysaccharide gels: different approaches for the determination of kinetic parameters. Trends Food Sci. Technol. 15(7-8): 394-402

Kallithraka S, Bakker J, Clifford MN, Vallis L (2001). Correlations between saliva protein composition and some T-I parameters of astringency. Food Quality and Preference 12(2): 145-152

Kopjar M, Andriot I, Saint-Eve A, Souchon I, Guichard E (2010). Retention of aroma compounds: an interlaboratory study on the effect of the composition of food matrices on thermodynamic parameters in comparison with water. Journal of the Science of Food and Agriculture 90(8): 1285-1292

Lasekan O (2013). A Comparative Analysis of the Influence of Human Salivary Enzymes on Odorant Concentration in Three Palm Wines. Molecules 18(10): 11809-11823

Lasekan O, Buettner A, Christlbauer M (2009). Investigation of the retronasal perception of palm wine (Elaeis guineensis) aroma by application of sensory analysis and exhaled odorant measurement (EOM). African Journal of Food, Agriculture, Nutrition and Development 9(2): 793-813

Linforth R, Martin F, Carey M, Davidson J, Taylor AJ (2002). Retronasal transport of aroma compounds. Journal of Agricultural and Food Chemistry 50(5): 1111-1117

Lubbers S, Decourcelle N, Vallet N, Guichard E (2004). Flavor release and rheology behavior of strawberry fatfree stirred yogurt during storage. Journal of Agricultural and Food Chemistry 52(10): 3077-3082

Mateus N, Pinto R, Ruao P, de Freitas V (2004). Influence of the addition of grape seed procyanidins to Port wines in the resulting reactivity with human salivary proteins. Food Chemistry 84(2): 195-200
McRae JM, Kennedy JA (2011). Wine and Grape Tannin Interactions with Salivary Proteins and Their Impact on Astringency: A Review of Current Research. Molecules 16(3): 2348-2364

Mishellany-Dutour A, Woda A, Laboure H, Bourdiol P, Lachaze P, Guichard E, Feron G (2012). Retro-Nasal Aroma Release Is Correlated with Variations in the In-Mouth Air Cavity Volume after Empty Deglutition. PLoS One 7(7): 8

Mitropoulou A, Hatzidimitriou E, Paraskevopoulou A (2011). Aroma release of a model wine solution as influenced by the presence of non-volatile components. Effect of commercial tannin extracts, polysaccharides and artificial saliva. Food Research International 44(5): 1561-1570

Mounayar R, Septier C, Chabanet C, Feron G, Neyraud E (2013). Oral fat sensitivity in humans: links to saliva composition before and after stimulation by oleic acid. Chemosens. Percept. 6(3): 118-126

Munoz-Gonzalez C, Martin-Alvarez PJ, Victoria Moreno-Arribas M, Angeles Pozo-Bayon M (2014). Impact of the Nonvolatile Wine Matrix Composition on the In Vivo Aroma Release from Wines. Journal of Agricultural and Food Chemistry 62(1): 66-73

Munoz-Gonzalez C, Rodriguez-Bencomo JJ, Moreno-Arribas MV, Pozo-Bayon MA (2011). Beyond the characterization of wine aroma compounds: looking for analytical approaches in trying to understand aroma perception during wine consumption. Analytical and Bioanalytical Chemistry 401(5): 1497-1512

Natera Marin R, Castro Mejias R, De Valme Garcia Moreno M, Garcia Rowe F, Garcia Barroso C (2002). Headspace solid-phase microextraction analysis of aroma compounds in vinegar. Validation study. Journal of chromatography. A 967(2): 261-7

Neyraud E, Palicki O, Schwartz C, Nicklaus S, Feron G (2012). Variability of human saliva composition: Possible relationships with fat perception and liking. Arch. Oral Biol. 57(5): 556-566

Overbosch P, Afterof WGM, Haring PGM (1991). Flavor release in the mouth. Food Reviews International 7(2): 137-184

Pierce J, Halpern BP (1996). Orthonasal and retronasal odorant identification based upon vapor phase input from common substances. Chemical Senses 21(5): 529-543

Pionnier E, Nicklaus S, Chabanet C, Mioche L, Taylor AJ, Le Quere JL, Salles C (2004). Flavor perception of a model cheese: relationships with oral and physico-chemical parameters. Food Quality and Preference 15(7-8): 843-852

Poette J, Lubbers S, Maison B, Andriot I, Pernin K, Guichard E, Cavellec A, Feron G (2010). The salivary reactor: an innovating tool for the categorization of food products through their aroma and taste compounds release profiles. In: T. Hofmann, W. Meyerhof and P. Schieberle (eds). Advances and challenges in flavor chemistry & biology. Proceedings of the 9th Wartburg Symposium. Deutsche Forschungsanstalt für Lebensmittelchemie, Freising (Germany), pp 386-389

Poette J, Mekoué J, Neyraud E, Berdeaux O, Renault A, Guichard E, Genot C, Feron G (2013). Fat sensitivity in humans: oleic acid detection threshold is linked to saliva composition and oral volume. Flavour Fragr. J.

Popp P, Paschke A (1997). Solid phase microextraction of volatile organic compounds using carboxen-polydimethylsiloxane fibers. Chromatographia 46(7-8): 419-424

Rabe S, Linforth RST, Krings U, Taylor AJ, Berger RG (2004). Volatile release from liquids: A comparison of in vivo APCI-MS, in-mouth headspace trapping and in vitro mouth model data. Chemical Senses 29(2): 163-173

Rinaldi A, Gambuti A, Moio L (2012). Precipitation of Salivary Proteins After the Interaction with Wine: The Effect of Ethanol, pH, Fructose, and Mannoproteins. Journal of Food Science 77(4): C485-C490

Robinson AL, Ebeler SE, Heymann H, Boss PK, Solomon PS, Trengove RD (2009). Interactions between Wine Volatile Compounds and Grape and Wine Matrix Components Influence Aroma Compound Headspace Partitioning. Journal of Agricultural and Food Chemistry 57(21): 10313-10322

Rodriguez-Bencomo JJ, Conde JE, Rodriguez-Delgado MA, Garcia-Montelongo F, Perez-Trujillo JP (2002). Determination of esters in dry and sweet white wines by headspace solid-phase microextraction and gas chromatography. Journal of Chromatography A 963(1-2): 213-223

Rodriguez-Bencomo JJ, Munoz-Gonzalez C, Andujar-Ortiz I, Jose Martin-Alvarez P, Victoria Moreno-Arribas M, Angeles Pozo-Bayon M (2011). Assessment of the effect of the non-volatile wine matrix on the volatility of typical wine aroma compounds by headspace solid phase microextraction/gas chromatography analysis. Journal of the Science of Food and Agriculture 91(13): 2484-2494

Salles C, Chagnon M-C, Feron G, Guichard E, Laboure H, Morzel M, Semon E, Tarrega A, Yven C (2011). In-Mouth Mechanisms Leading to Flavor Release and Perception. Critical Reviews in Food Science and Nutrition 51(1): 67-90

Simon C, Barathieu K, Laguerre M, Schmitter JM, Fouquet E, Pianet I, Dufourc EJ (2003). Three-dimensional structure and dynamics of wine tannin-saliva protein complexes. A multitechnique approach. Biochemistry 42(35): 10385-10395

Spedding PL, Grimshaw J, Ohare KD (1993). Abnormal evaporation rate of ethanol from low concentration aqueous-solutions. langmuir 9(5): 1408-1413

Taylor AJ (1996). Volatile flavor release from foods during eating. Critical Reviews in Food Science and Nutrition 36(8): 765-784

Tromelin A, Andriot I, Kopjar M, Guichard E (2010). Thermodynamic and Structure Property Study of Liquid-Vapor Equilibrium for Aroma Compounds. Journal of Agricultural and Food Chemistry 58(7): 4372-4387

Tsachaki M, Gady A-L, Kalopesas M, Linforth RST, Athes V, Marin M, Taylor AJ (2008). Effect of ethanol, temperature, and gas flow rate on volatile release from aqueous solutions under dynamic headspace dilution conditions. Journal of Agricultural and Food Chemistry 56(13): 5308-5315

van Ruth SM, Buhr K (2003). Influence of saliva on temporal volatile flavour release from red bell peppers determined by proton transfer reaction-mass spectrometry. European Food Research and Technology 216(3): 220-223

van Ruth SM, Grossmann I, Geary M, Delahunty CM (2001). Interactions between artificial saliva and 20 aroma compounds in water and oil model systems. Journal of Agricultural and Food Chemistry 49(5): 2409-2413

vanRuth SM, Roozen JP, Nahon DF, Cozijnsen JL, Posthumus MA (1996). Flavour release from rehydrated French beans (Phaseolus vulgaris) influenced by composition and volume of artificial saliva. Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung 203(1): 1-6

Vasserot Y, Arnaud A, Galzy P (1993). Evidence for muscat marc monoterpenol glucosides hydrolysis by free or immobilized yeast beta-glucosidase. bioresource technology 43(3): 269-271

voirol e, daget n (1986). Comparative-study of nasal and retronasal olfactory perception. Lebensmittel-Wissenschaft & Technologie 19(4): 316-319

Yang SS, Huang CB, Smetena I (2002). Optimization of headspace sampling using solid-phase microextraction for volatile components in tobacco. Journal of Chromatography A 942(1-2): 33-39

4. 3. 2 Papel de la mucosa oral en la retención y posterior liberación de compuestos del aroma del vino.

Durante el consumo de alimentos, la mayor parte de los compuestos volátiles se pierden en el esófago durante la ingestión de los mismos. El resto, queda adherido a las mucosas faríngea y oral es transportado, debido al flujo respiratorios contracorriente, o lo que es lo mismo, en la primera exhalación tras la deglución a los órganos olfativos (Linforth & Taylor, 2006). Por tanto, el papel que ejercen las mucosas orales en la retención de compuestos volátiles durante el consumo de alimentos es clave a la hora de entender la percepción retronasal y, sobre todo, la persistencia de aroma.

En el caso del vino, estos estudios apenas si han sido abordados, aunque hay evidencias científicas que sugieren un posible papel de las mucosas en la diferencias a nivel de persistencia entre vinos (Buettner). Es por ello que se planteó este trabajo con el objetivo de evaluar la capacidad de retención de las mucosas orales de compuestos característicos del aroma del vino, y en segundo lugar evaluar la capacidad de liberación de los compuestos adsorbidos teniendo en cuenta diferencias en la composición de la matriz vínica. En experimentos preliminares se ha procedido a cuantificar los odorantes retenidos por la mucosa oral mediante dos técnicas: la técnica SOOM (spit off odorant measurement) consiste en calcular la diferencia entre la cantidad de aroma presente en una solución aromatizada mantenida en la boca durante cierto tiempo y la cantidad de aroma en el expectorado. A su vez, para monitorizar el aroma liberado de la mucosa tras la expectoración, se optimizó una técnica basada en la monitorización intraoral *in vivo* - HS-SPME-GC/MS. Se evaluaron diferentes parámetros que podrían condicionar la cantidad de aroma adsorbido a la mucosa, como el efecto del tiempo de residencia del vino en la boca, o la concentración de compuesto. Además la cinética de liberación del aroma adsorbido se realizó a distintos tiempos, y se consideraron las diferencias interindividuales.

A continuación se presentan los resultados de este trabajo en forma de publicación científica, pese a que es un estudio en curso:

Publicación 6. Papel de la mucosa oral en la retención y posterior liberación de compuestos del aroma del vino (Manuscrito en preparación).

Introduction

In recent years, a lot of research has been addressed toward understanding retronasal aroma perception that is a key modulator for food consumption and consumer's preferences (Gierczynski et al. 2011; Mishellany-Dutour et al. 2012). The perception of aroma during food consumption is a complex phenomenon involving the release of odorants within the oral cavity from the food material, their transport via the retronasal route to the nasal cavity, followed by the interaction of odorants with the respective receptors in the olfactory epithelium and the subsequent transduction of the sensory signals to the brain. During the consumption of liquid foods (such as wine) the major part of aroma compounds reach the olfactory receptors after swallowing like a pulse of aroma, usually called 'swallow-breath' (Land 1996; Buettner and Schieberle 2000). This is due to the formation of a thin layer of the liquid sample on the surface of the pharynx acting as an aroma reservoir ready to be released by the expiration flows, and this has been visualized by videofluoroscopy when a volunteer swallowed viscous oral contrast medium (Buettner et al. 2002). However, additional aroma peaks could be perceived by further swallowing actions of saliva and a proportion of the flavor-enriched liquid remains in the mouth and pharynx as a film coating.

Therefore, two key modes of aroma release and perception have to be distinguished. The immediate aroma impression, when liquid food is just swallowed, and the prolonged retronasal aroma perception after swallowing, often called the after-odour (Buettner 2004). The opposite effect, with odorants being no longer present in the oral cavity but being still perceived due to cognitive or receptor phenomena, cannot be excluded but has, to our knowledge, not yet been shown (Buettner 2004). After-odour perception is influenced by a series of physiological and physicochemical parameters, as shown previously (Buettner et al. 2001; Buettner et al. 2002). One of these key parameters is the adsorptive potency of odorants (Lasekan et al. 2009) or non-odour aroma precursor compounds to oral mucosa. In the last case, odorants need to be newly generated from the aroma precursors due to the enzymatic metabolism by oral microbiota (Starkenmann et al. 2008); Muñoz-Gonzalez et al. 2014a). The adsorption of

odorants, as well as the adsorption of food matrix material, has been previously shown to occur (Buettner et al. 2002).

In spite of the relevance of this area, there are not many scientific works directed toward understanding the aroma release during wine consumption, and traditionally the investigations in this field had focused on the characterization of the wine volatile profile (Muñoz-Gonzalez et al. 2011). To study wine aroma release during consumption, different *in vivo* (Munoz-Gonzalez et al. 2014) and *in vitro* (Genovese et al. 2009; Muñoz-Gonzalez et al. 2014) approaches have been recently proposed. However, little is known about the persistence of volatile compounds after wine consumption, and only limited to some general sensory descriptions. For example, (Goodstein Emily S. 2014) have performed a time-intensity study and they have observed differences in the persistence of some aromatic notes in model white wine. It seems like fruity notes are less persistent than coco, mushrooms or floral notes.

Analytical study of odorants responsible for aroma persistence could be monitored *in vivo* by using different trapping devices (Buettner 2004) or spectrometric techniques (APCI-MS, PTR-MS) (Linforth and Taylor 2000) and several works assume the existence of aroma compounds/mucosa interactions in aqueous solutions (Buettner and Welle 2004), wine (Buettner 2004) and palm wine (Lasekan 2013). However to our knowledge, there is no understanding of the effect that wine matrix could exert on these interactions. In addition, it is well known the high interindividual variability in the amount of aroma compounds release between subjects (Pionnier et al. 2004; Gierczynski et al. 2011; Munoz-Gonzalez et al. 2014). Nevertheless, there are no studies focused on investigate inter-individual differences on aroma persistence, which could explain the differences observed *in vivo* aroma perception after consumption.

The aim of this study is to develop experimental protocols in order to characterize and assess *in vivo* the interactions existing between wine aroma compounds and oral mucosa that can account for persistence phenomena during wine consumption.

Material and methods

Wine samples

Two commercial non-aromatic wines (white and red) were selected for this study. Aromatization was performed with a mixture of six food-grade aroma compounds (Sigma-Aldrich, Steinheim, Germany) representative of the wine volatile profile (ethyl hexanoate, β-ionone, linalool, guaiacol, β-phenylethanol and isoamyl acetate), all of them characterized for having a wide range of physicochemical properties (**Table 1**). To do that, six independently aroma stock solutions in ethanol absolute were prepared and from there, each aroma compound was added to the wines to obtain the different concentrations depending on the experiment (0.5, 1, 1.5 or 2 mg L^{-1}).

Table 1. Physicochemical properties of the aroma compounds employed in this study.

Compound	CAS number	MW (g mol^{-1})	BP (ºC)	log *P* [(a)]	Descriptor[(b)]
Isoamyl acetate	123-92-2	130	134	2.26	banana
Ethyl hexanoate	123-66-0	144	167	2.83	apple peel, fruit
Linalool	78-70-6	152	204	3.38	flower, lavender
Guaiacol	90-05-1	124	211	1.34	spice, clove
β-Phenylethanol	60-12-8	122	224	1.57	honey, spice, rose
β-Ionone	8013-90-9	192	262	4.42	raspberry, violet, flower,

(a) log *P* = log of the water partition coefficient estimated from molecular modeling software EPI Suit (U.S EPA 2000-2007).

(b) From Flavornet (http://www.flavornet.org; accessed October 2009) database, from NIST web chemistry book (2005) (http://www.webbook.nis.gov/chemistry).

Panelists

Three volunteers (females) between 26-39 years old previously trained in the aroma trapping procedure participated in this study. They were instructed not to eat, drink or smoke 2 hours before the experiments. They had no known illnesses and had self-reported normal olfactory and gustatory functions. 15 minutes before each experiment, the panelists had to clean their mouths and rinse with a bicarbonate solution and water. The monitoring of the oral cavity of the panelists for the six compounds of interest was performed before each analysis**.**

Mouth rinsing

The procedure for the mouth rinsing was based on that described by Buettner et al., (2002) with some modifications. 15 ml of the aromatized wine (total amount of each odorant: 0.015 mg) was taken into the oral cavity, kept for 15, 30 or 60 s in order to favor the equilibration of the aroma compounds within the oral cavity, then expectorated. During rinsing, care was taken to keep the lips closed, not to swallow and not to open the velum – tongue border prior to expectoration. Analyses were performed two times by two panelists. After expectorations, a saturated $CaCl_2$-solution was added to inhibit enzymic reactions, and the spit-off samples were kept at -20 ºC until extraction.

For the extraction procedure, 10 mL of the spit-off wine solutions were spiked 10 µl of the internal standard, methyl nonanoate (1 mg L^{-1}). The solutions were extracted with 1 mL of dichloromethane (2*x*), then ultrasonicated (15 min) and finally centrifuged (5000 rpm, 4 ºC, 15 min) to separate the two phases. The combined organic extracts were dried over anhydrous Na_2SO_4, then concentrated to a total volume of 500 µl and subsequently analysed by gas chromatography/mass spectrometry. Prior to the concentration, 50 µl of the internal standard 3-octanol (1 mg L^{-1}) were added. The same extraction procedure was followed with the original aromatized wine, and allowed calculate the percentage of adsorption by the oral mucosa. A previously determined volume of fresh saliva was added in order to avoid the interaction effect of salivary proteins (Muñoz-Gonzalez, submitted).

$$\%\ Aroma\ Adsorption = \frac{a_o - a_{exp}}{a_o}\ x\ 100$$

Where a_o is the aroma present in the aromatized wine without mouth rinsing, and $a_{exp}$is the aroma present in the wines after the mouth rinsing.

Two µL of the concentrated extract was injected in splitless mode in the injector port of a Gas Chromatograph Agilent 6890N coupled to a quadrupole Mass Detector Agilent 5973.

Intraoral Sampling of Odorants

At defined time intervals after expectoration (30, 60, 120 s, 300 s), a DVB/CAR/PDMS (Divinylbenzene/Carboxen/Polydimethylsiloxane 50/30 µm

thickness -2 cm length-) coated SPME fiber (Supelco, Bellefonte, PA) SPME fiber with a home-made adaptor was placed into the oral cavity. After 2 min of equilibration, the fiber was removed from the oral cavity, and immediately placed into the split/splitless injector. During extraction, the lips and velum were kept closed and it will be avoiding make swallowing actions.

Desorption was performed in the injector of the GC system (Agilent 6890N) in splitless mode for 1.5 min at 270 ºC. After each injection the fiber was cleaned for 30 min to avoid any memory effect. Analyses were performed three times by each of the three panelists.

GC/MS analysis

The identification of volatile compounds was carried out with a Gas Chromatograph Agilent 6890N coupled to a quadrupole Mass Detector Agilent 5973. The injection temperature was set at 270 ºC. Volatile compounds were separated on a DB-Wax polar capillary column (60 m × 0.25 mm i.d. × 0.50 µm film thickness) from Agilent (J&W Scientific, Folsom, USA). Helium was the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature was initially held at 40 ºC for 2 min, then increased at 8 ºC min^{-1} to 240 ºC and held for 15 min.

For the MS system (Agilent 5973N), the temperature of the transfer line, quadrupole and ion source were 270, 150 and 230 ºC respectively. Electron impact mass spectra were recorded at 70 eV ionization voltages and the ionization current was 10 µA. The acquisitions were performed in Scan (from 35 to 350 amu) and SIM modes. The identification of compounds was based on the comparison of retention times and mass spectra. The mass spectra were compared with those from NIST 2.0 database. Relative peak areas (RPAs) were obtained by calculating the relative peak area in relation to that of the internal standard. The use of RPAs data to express aroma release was sufficient for this type of analysis as the aim of the work was to compare the extent of aroma release between wine samples. Response factors (RFs) in the MS were calculated by injecting increased concentrations (from 1 to 20 mg L^{-1}) of a mixture of the five aroma compounds (all at the same concentration) using the same chromatographic conditions described above. The calculated RFs were: 12319, 12024, 3849, 10956, 4740 and 27726 for isoamyl acetate, ethyl hexanoate, linalool, guaicol, β-phenyl ethanol and β-ionone respectively.

Statistical analysis

One way ANOVA was used to determine the effect of mouth rinsing. Two way ANOVA was employed to evaluate significant differences on the release among compounds and panelists. Least significant difference (LSD) test was used for mean comparison. The STATISTICA program for Windows version 7.1 was used for data processing (StatSoft, Inc., 2005, www.statsoft.com).

Results and discussion

Adsorption of odorants to the oral mucosa: Mouth rinsing

During wine drinking, there are two different places in which odorants can be found: in the throat, forming a thin film, and in the mouth, forming a thicker film (Ferreira et al., 2006). Therefore, the mouth is a major reservoir for volatiles ready to be released after consumption contributing to the 'afterodor' perception. By application of the SOOM technique, the adsorptive behavior of odorants to oral mucosa at different times (15, 30, 60 s) was calculated as explained in the M&M section and the results are shown in **Table 2**.

Table 2. Percentage of aroma compounds adsorbed to oral mucosa after mouth rinsing with aromatized wine during different times (15 s, 30 s, 60 s).

	% adsorption		
	15 s	30 s	60 s
Isoamyl acetate	7,07	26,19	16,30
Ethyl hexanoate	33,26	45,47	41,32
Linalool	25,50	37,99	35,57
Guaiacol	33,36	45,41	44,31
β-phenylethanol	0	9,92	1,90
β -Ionone	36,38	46,89	44,62

As can be seen in the table, the percentage of adsorption was dependent on the physicochemical characteristics of the aroma compound and ranged from 0 to 47 %. In general, β-ionone, ethyl hexanoate, linalool and guaiacol were highly retained (> 40 %). Among them, β-ionone, ethyl hexanoate and linalool were the compounds with high molecular weight and log *P* values. This seems indicate that the more hydrophobic compounds could be more susceptible to the adsorption by the oral mucosa which might

be related with the presence of hydrophobic interactions between these compounds and the oral mucosa. One possible explanation is that saliva coats all surfaces in the mouth forming a salivary film. On the mucosa the pellicle it is formed with salivary proteins directly binding to the oral epithelium and being stabilized by protein cross-linking (Bradway et al. 1992), forming the called salivary mucosal pellicle (Carpenter 2013). Among the functions of the salivary mucosal pellicle could be its contribution to lubrication, helping to prevent abrasion between surfaces, improving swallowing abilities and contributing to a normal mouthfeel (Humphrey and Williamson 2001). However, these saliva proteins could also establish hydrophobic interactions with wine aroma compounds that would explain our results.

However, guaiacol (the compound more polar assayed) presented also a high retention. This aromatic compound showed also a high boiling point, which could explain this retention effect.

In the other hand, isoamyl acetate and β-phenylethanol were the compounds less retained and they presented low log *P* values. Specifically, β-phenylethanol (polar and aromatic) did not suffer retention at 15 s, while at 30 s was lower than 10 %.

In this regard, the adsorption effect seems to be dependent on the residence time in the mouth, according with Weel et al. (Weel et al. 2003). As it is shown in the **Table 2** in general, the lowest adsorption aroma values were observed during the mouth-rinsing of 15 seconds, which means a lower retention of these compounds in human mucosa. However, at 30 and 60 seconds of the mouth rinsing no significative differences were observed in the adsorption values (data no shown), so 30 s of mouth rinsing was selected because it is a shorter time and therefore could be more comparable to the real consumption situation.

After selecting 30 s as the optimal time of mouth rinsing we checked the inter- and intra-variability of the methodology (**Table 3**). Three panelists carried out the procedure 8 different times. The intra-variability (n = 8) was lower than 10 % for all the compounds assayed. The inter-variability (n = 3) was also lower than 3 % for most of the aroma compounds and only β-ionone values were significantly different among panelists (data no shown). This is the compound most hydrophobic assayed in this experiment, and, as previously suggested, the one more affected to the mucosa adsorption, which might explain its susceptibility to show differences among panelists.

Table 3. Average of relative areas and coefficients of variation (CV) for all the compounds assayed in this experiment (30 s mouth rinsing). repetitibility (*n*=8).

	Panelist 1		Panelist 2		Panelist 3		Interindividual differences
	Average	CV (%)	Average	CV (%)	Average	CV (%)	CV (%)
Isoamyl acetate	2,27	7,69	2,16	10,61	2,26	7,30	2,83
Ethyl hexanoate	0,53	5,91	0,51	7,52	0,52	8,04	1,88
Linalool	0,30	5,04	0,30	6,26	0,32	4,95	2,68
Guaiacol	0,45	4,07	0,46	7,20	0,46	4,31	1,57
β-Phenylethanol	3,90	5,49	4,04	2,98	4,07	6,68	2,24
β-Ionone	0,56	8,18	0,65	5,93	0,65	9,47	7,93

As previously suggested by Buettner et al.(Buettner et al. 2001) the relatively high decrease of the most of the compounds during their exposition to the oral cavity cannot be explained simply by partitioning from the water to the gas phase present in the oral cavity. Therefore, we can conclude that the major amount of the odorants lacking in the spit-off solutions were indeed bound in the oral cavity by adsorption or were even resorbed via the oral mucosa.

Aroma release from the oral mucosa: Intraoral Sampling of Odorants

Kinetic of aroma release of the compounds adsorbed to the human mucosa within the mouth

Once the optimal time of mouth rinsing (30 s) was selected, the kinetic of aroma release of the compounds adsorbed to the human mucosa within the mouth was performed. To do that, 3 panelists monitorized the volatile release in their mouths with a SPME fiber at different times (30, 60, 120 and 300 s). Aroma release data were submitted to a two-way ANOVA which showed that all the compounds were statically influenced by both panelist (except linalool) and time, and their interaction (data no shown).

Figure 1 shows the example of the kinetic release for the three panelists, together with the results of the LSD test. As can be seen, all the compounds undergo a gradual release over time. In general longer times indicated lower release. However all

the compounds were detected after 300 s which could be interesting in the basis of explaining aroma persistence (after-odor) during wine consumption.

As can be seen, in general, panelists 2 y 3 showed similar patterns of aroma release while panelist 1 showed the most different behavior. This seemed indicate that the volatile adsorbed can be further released from the mucosa at different rates depending on individual differences. Among them, the breathing flows could be an important factor that drives aroma release, as previously shown (Pionnier et al. 2004; Frank et al. 2011; Munoz-Gonzalez et al. 2014).

In spite of the interindividual variation, differences were also dependent of the aroma chemical class. As shown in **Figure 1**, the two esters (isoamyl acetate and ethyl hexanoate) were highly released at first, but their concentration decreased rapidly. This means that these compounds associated with fruity aromatic notes could be not very persistent. This observation has been recently highlighted by a sensorial time-intensity study in model white wines (Goodstein et al. 2014). One possible reason to that might be the degradation of esters by hydrolysis and aldehyde-reduction, as many esterolytic enzymes can be found in human saliva (Chauncey et al. 1954), and as odorant metabolism in the presence of saliva has been reported previously in water solutions (*in vitro* conditions) (Buettner 2002; Buettner 2002). However, other possible reason could be related with the volatility of these two compounds. As previously shown by Linforth and coworkers (Linforth et al. 2002); (Buettner 2004); (Espinosa-Diaz 2004), and Ferreira et al. (Ferreira et al. 2006) the volatility plays an important role in determining retronasal release of odorants. In general, lower volatilities (high boiling points) are correlated with higher aroma persistence. This observation is in accordance with our results since the compounds with the lowest boiling points assayed (esters) showed the most rapid decrease.

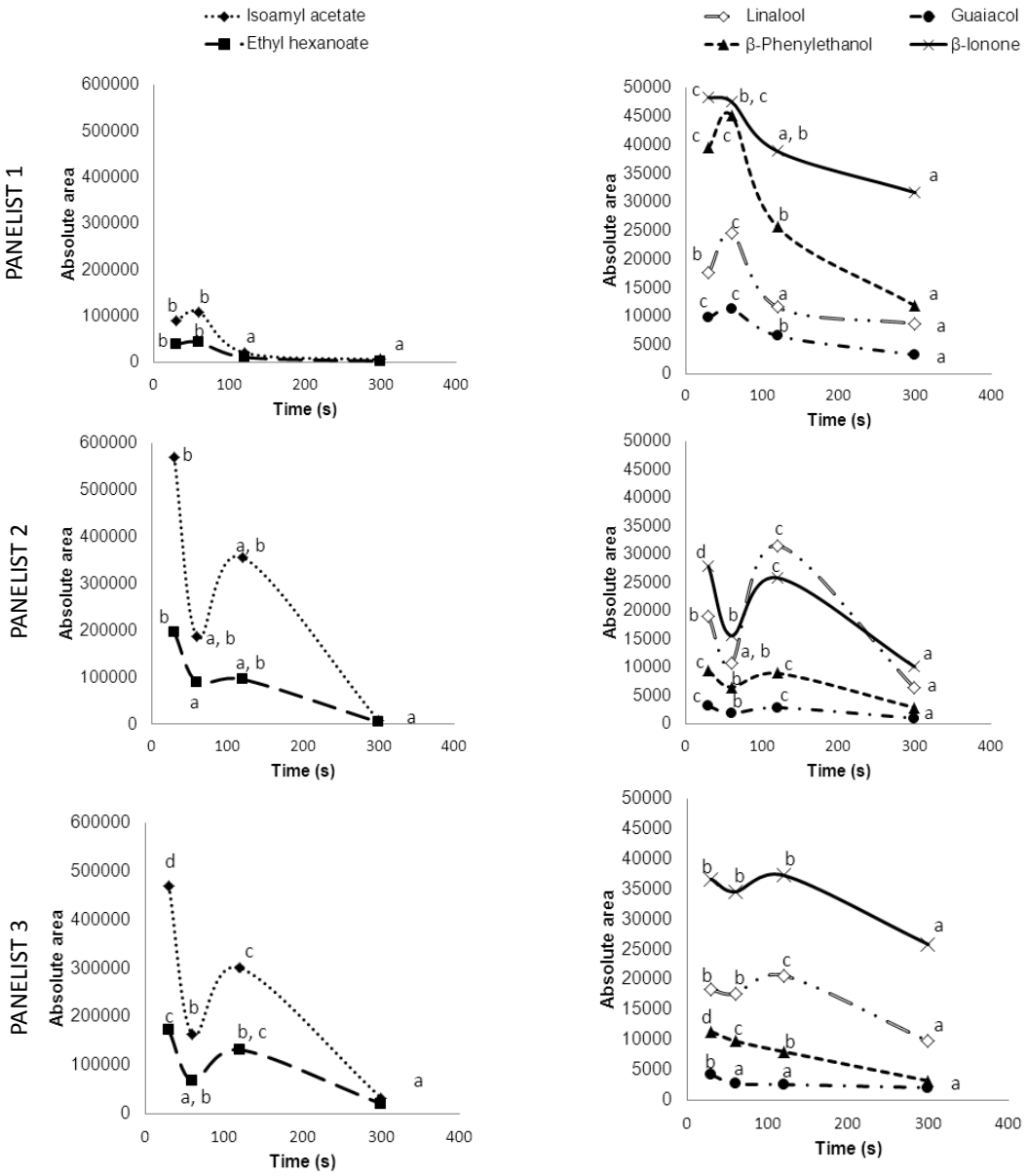


Figure 1. Kinetic of the aroma release adsorbed to the oral mucosa.

However, more hydrophobic compounds, such as β-ionone, linalool, as well as the polar β-phenylethanol and guaiacol, seemed to be released more slowly and constant throughout the time. All of these molecules showed the highest boiling points which is in accordance with the previous explanation. Moreover these results have been recently confirmed by Goodstein et al. (2014), who observed a high persistence of flowery notes in model white wine (**Table 1**). These results were also accorded with Lasekan et al.

(2009) who investigated the aroma persistence of odorants from palm wine and found that linalool was more persistent than ethyl hexanoate, although the methodology and the times employed were different.

Interestingly, guaiacol decreased rapidly in the panelist 3 while more slowly in the panelists 1 and 2. This could be due to a metabolization of this compound by the action of some enzymes presents in human saliva, and highlights the interindividual variations in salivary composition.

Effect of Wine Matrix Composition on Aroma Persistence

To evaluate the effect of the wine non-volatile matrix composition on the aroma release after mouth rinsing during 30 s, regression lines for the 6 volatile compounds using three replicates at four levels of concentration for each of the wines (white and red) were calculated. The effect of wine matrix composition on aroma release was different depending on the compound and on the concentration assayed (**Figure 2**). This effect was more evident for isoamyl acetate and β-phenylethanol.

In general, red wine showed the lowest aroma released values (except for the β-phenylethanol). This might be due to the formation of complexes among the wine polyphenols (more abundant in red wines) and salivary proteins, which could retain aroma molecules, resulting in a minor aroma release. In these experimental conditions, human mouth acts as a kind of closed reactor (no airflows), which could simulate static conditions. As previously shown in two *in vitro* study which leading to evaluate the effect of saliva on aroma release, the formation of this types of complexes resulting in a retention effect (Mitropoulou et al. 2011; Muñoz-Gonzalez et al. 2014). However, these complexes could be retain in the salivary mucosal pellicle, as an aroma reservoir that could promote a high aroma release in red wines in a dynamic situation, as previously shown *in vitro* (Muñoz-Gonzalez et al. 2014) and *in vivo* (Munoz-Gonzalez et al. 2014).

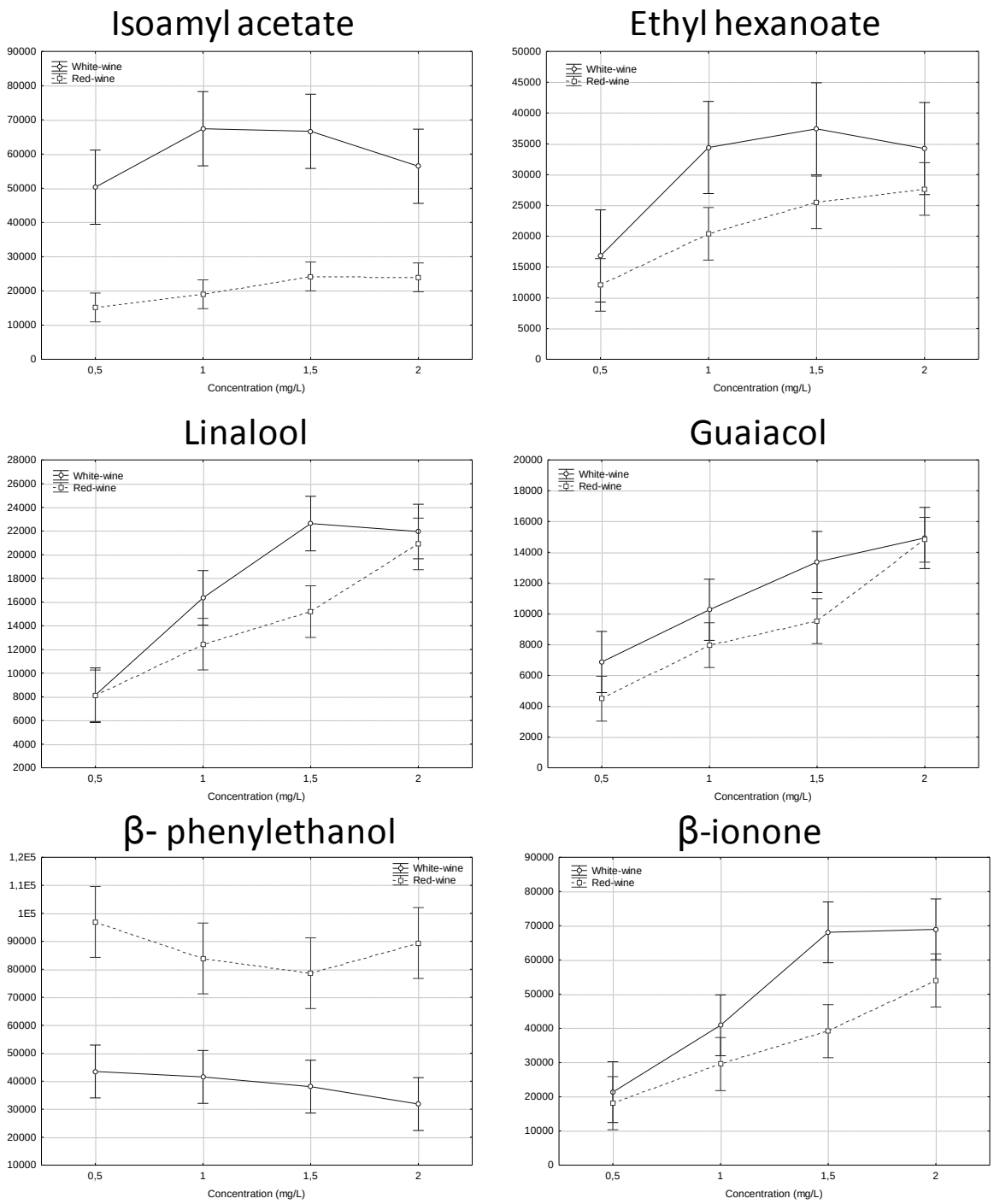


Figure 2. Results of the aroma release after the mouth rinsing with the white and red wines.

White-wine Red-wine

References

Bradway SD, Bergey EJ, Scannapieco FA, Ramasubbu N, Zawacki S, Levine MJ (1992). Formation of salivary-mucosal pellicle - the role of transglutaminase. Biochemical Journal 284: 557-564

Buettner A (2002). Influence of human saliva on odorant concentrations. 2. aldehydes, alcohols, 3-alkyl-2-methoxypyrazines, methoxyphenols, and 3-hydroxy-4,5-dimethyl-2(5H)-furanone. Journal of Agricultural and Food Chemistry 50(24): 7105-7110

Buettner A (2002). Influence of human salivary enzymes on odorant concentration changes occurring in vivo. 1. Esters and thiols. Journal of Agricultural and Food Chemistry 50(11): 3283-3289

Buettner A (2004). Investigation of potent odorants and afterodor development in two Chardonnay wines using the buccal odor screening system (BOSS). Journal of Agricultural and Food Chemistry 52(8): 2339-2346

Buettner A, Beer A, Hannig C, Settles M (2001). Observation of the swallowing process by application of videofluoroscopy and real-time magnetic resonance imaging-consequences for retronasal aroma stimulation. Chemical Senses 26(9): 1211-1219

Buettner A, Beer A, Hannig C, Settles M, Schieberle P (2002). Physiological and analytical studies on flavor perception dynamics as induced by the eating and swallowing process. Food Quality and Preference 13(7-8): 497-504

Buettner A, Schieberle P (2000). Exhaled odorant measurement (EXOM) — a new approach to quantify the degree of in-mouth release of food aroma compounds. Lebensmittel-Wissenschaft und-Technologie 33(8): 553-559

Buettner A, Welle F (2004). Intra-oral detection of potent odorants using a modified stir-bar sorptive extraction system in combination with HRGC-0 known as the buccal odor screening system (BOSS). Flavour and Fragrance Journal 19(6): 505-514

Carpenter GH (2013). The secretion, components and properties of saliva. Annual Review of Food Science and Technology 4: 267-276

Chauncey HH, Lionetti F, Winer RA, Lisanti VF (1954). Enzymes of human saliva .1. the determination, distribution, and origin of whole saliva enzymes. journal of dental research 33(3): 321-334

Espinosa-Diaz M (2004). Comparison between orthonasal and retronasal flavour perception at different concentrations. Flavour and Fragrance Journal 19(6): 499-504

Ferreira V, Petka J, Cacho J (2006). Intensity and persistence profiles of flavor compounds in synthetic solutions. Simple model for explaining the intensity and persistence of their aftersmell. Journal of Agricultural and Food Chemistry 54(2): 489-496

Frank D, Appelqvist I, Piyasiri U, Wooster TJ, Delahunty C (2011). Proton Transfer Reaction Mass Spectrometry and Time Intensity Perceptual Measurement of Flavor Release from Lipid Emulsions Using Trained Human Subjects. Journal of Agricultural and Food Chemistry 59(9): 4891-4903

Genovese A, Piombino P, Gambuti A, Moio L (2009). Simulation of retronasal aroma of white and red wine in a model mouth system. Investigating the influence of saliva on volatile compound concentrations. Food Chemistry 114(1): 100-107

Gierczynski I, Guichard E, Laboure H (2011). Aroma perception in dairy products: the roles of texture, aroma release and consumer physiology. A review. Flavour and Fragrance Journal 26(3): 141-152

Goodstein Emily S. BJC, Evans Marc, Ross Carolyn F. (2014). Perception of flavor finish in model white wine: A time-intensity study. Food Quality and Preference 36: 50-60

Humphrey SP, Williamson RT (2001). A review of saliva: Normal composition, flow, and function. Journal of Prosthetic Dentistry 85(2): 162-169

Land DG (1996). Perspectives on the effects of interactions on flavor perception: An overview. In: R. J. McGorrin and J. V. Leland (eds). Flavor-Food Interactions. pp 2-11

Lasekan O (2013). A Comparative Analysis of the Influence of Human Salivary Enzymes on Odorant Concentration in Three Palm Wines. Molecules 18(10): 11809-11823

Lasekan O, Buettner A, Christlbauer M (2009). Investigation of the retronasal perception of palm wine (Elaeis guineensis) aroma by application of sensory analysis and exhaled odorant measurement (EOM). African Journal of Food, Agriculture, Nutrition and Development 9(2): 793-813

Linforth R, Martin F, Carey M, Davidson J, Taylor AJ (2002). Retronasal transport of aroma compounds. Journal of Agricultural and Food Chemistry 50(5): 1111-1117

Linforth R, Taylor AJ (2000). Persistence of volatile compounds in the breath after their consumption in aqueous solutions. Journal of Agricultural and Food Chemistry 48(11): 5419-5423

Mishellany-Dutour A, Woda A, Laboure H, Bourdiol P, Lachaze P, Guichard E, Feron G (2012). Retro-Nasal Aroma Release Is Correlated with Variations in the In-Mouth Air Cavity Volume after Empty Deglutition. Plos One 7(7)

Mitropoulou A, Hatzidimitriou E, Paraskevopoulou A (2011). Aroma release of a model wine solution as influenced by the presence of non-volatile components. Effect of commercial tannin extracts, polysaccharides and artificial saliva. Food Research International 44(5): 1561-1570

Munoz-Gonzalez C, Martin-Alvarez PJ, Victoria Moreno-Arribas M, Angeles Pozo-Bayon M (2014). Impact of the Nonvolatile Wine Matrix Composition on the In Vivo Aroma Release from Wines. Journal of Agricultural and Food Chemistry 62(1): 66-73

Muñoz-Gonzalez C, Feron G, Guichard E, Rodriguez-Bencomo J, Alvarez PM, Moreno-Arribas M, Pozo-Bayon M (2014). Understanding the role of saliva on aroma release from wine by using static and dynamic headspace conditions Submitted

Carolina Muñoz-González, Juan J. Rodríguez-Bencomo, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón. Feasibility and application of a retronasal aroma-trapping device to study in vivo aroma release during the consumption of model wine-derived beverages. Food Science and Nutrition, 2014 DOI: 10.1002/fsn3.111

Carolina Muñoz-González, Etienne Semon, Gilles Feron, Elisabeth Guichard, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón (2014). Wine matrix composition affects temporal aroma release as measured by PTR-ToF-MS. Submitted

Muñoz-Gonzalez C, Cueva C, Pozo-Bayon M A, Moreno-Arribas M V (2014). Ability of human oral microbiota to produce wine odorant aglycones from odorless grape glycosidic aroma precursors. Submitted

Muñoz-Gonzalez C, Rodriguez-Bencomo JJ, Moreno-Arribas MV, Pozo-Bayon MA (2011). Beyond the characterization of wine aroma compounds: looking for analytical approaches in trying to understand aroma perception during wine consumption. Analytical and Bioanalytical Chemistry 401(5): 1497-1512
Pionnier E, Chabanet C, Mioche L, Le Quere JL, Salles C (2004). 1. In vivo aroma release during eating of a model cheese: Relationships with oral parameters. Journal of Agricultural and Food Chemistry 52(3): 557-564

Starkenmann C, Le Calve B, Niclass Y, Cayeux I, Beccucci S, Troccaz M (2008). Olfactory perception of Cysteine- S-Conjugates from fruits and vegetables. Journal of Agricultural and Food Chemistry 56(20): 9575-9580

Weel KGC, Boelrijk AEM, Burger JJ, Claassen NE, Gruppen H, Voragen AGJ, Smit G (2003). Effect of whey protein on the in vivo release of aldehydes. Journal of Agricultural and Food Chemistry 51(16): 4746-4752

4. 3. 3 Efecto de la microbiota bacteriana de la cavidad oral en la generación de moléculas odorantes a partir de precursores no odorantes de la uva.

Entre los factores relacionados con la fisiología oral que podrían afectar la composición del aroma durante el consumo, el papel de los microorganismos de la cavidad oral es prácticamente desconocido. No obstante, investigaciones recientes, (Starkenman y col., 2008; Mayr y col., 2014) sugieren que la microbiota oral podría ser capaz de hidrolizar precursores de aroma liberando las correspondientes agliconas odorantes, lo que podría estar está relacionado con la persistencia de aroma durante el consumo.

En el caso específico del vino, el perfil aromático puede estar determinado en muchos casos por los compuestos varietales de las uvas que se han empleado en su elaboración. Estos compuestos pueden estar presentes tanto en su forma libre como conjugados en forma de precursores glicosídicos, que pueden llegar a constituir una importante reserva de aroma en los vinos.

Por ello, el pso siguiente consistió en evaluar la capacidad de la microbiota para generar moléculas odorantes a partir de precursores glicosidicos aislados previamente de uvas. Para ello, fue necesario disponer de técnicas de aislamiento de este tipo de compuestos tanto de uvas como de orujos de uvas. Entre ellas, la técnica convencional de extracción líquido-líquido ha sido ampliamente utilizada (Hernandez-Orte y col., 2009). Además, evaluamos la posibilidad de emplear otra técnica de extracción como la extracción con líquidos presurizados (PLE) que ha demostrado ser eficaz para extraer diferentes fitoquímicos de plantas. La comparación de ambas técnicas se llevó a cabo empleando como materia prima subproductos de vinificación, cuya acumulación supone además un grave problema medioambiental. Estos resultados dieron lugar a la **Publicación 7**.

Los extractos obtenidos con las diferentes metodologías se utilizaron para evaluar el papel de microbiota bacteriana oral en la liberación de compuestos de aroma. Sin embargo, en experimentos previos de viabilidad bacteriana observamos que el extracto obtenido mediante PLE inhibía el crecimiento de las bacterias orales, probablemente debido a su alto contenido en polifenoles, así que se decidió seguir trabajando con el extracto de precursores obtenido por maceración estática. Estos

resultados se recogen en la **Publicación 8**. En primer lugar, se evaluó la capacidad de hidrólisis de nueve bacterias representativas de la cavidad oral y en su segundo experimento, se evaluó el papel del conjunto de la microbiota de la cavidad oral aislada a partir de saliva de voluntarios sanos en condiciones aerobias y anaerobias. Las agliconas odorantes liberadas se determinaron mediante HS-SPME-GC/MS.

A continuación se presentan los resultados de estos trabajos en forma de publicaciones científicas:

Publicación 7: **Carolina Muñoz-González**, Juan J. Rodríguez-Bencomo, Pedro J. Martín-Álvarez, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón. "Recovery of aromatic aglycones from grape pomace winemaking by-products by using liquid-liquid and pressurized-liquid extraction". *Food Analytical Methods*, 7 (1) **2014** 47-57.

- Además este trabajo fue presentado como comunicación oral titulado "Recovery of aromatic aglycones from winemaking by-products" en la VIII International Conference on Enoforum, Arezzo, Italy, 7-9 may, 2013. **Carolina Muñoz-González**, Juan J. Rodríguez-Bencomo, Pedro J. Martín-Álvarez, José A. Mendiola, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón.

Publicación 8: Carolina Muñoz-González, Carolina Cueva, M. Ángeles Pozo-Bayón, M. Victoria Moreno-Arribas. "Ability of human oral microbiota to produce wine odorant aglycones from odorless grape glycosidic aroma precursors". Publicación enviada.

- Además una parte de este trabajo fue presentado como comunicación tipo poster titulado "Ability of oral microbiota to release free volatiles from wine odorless glycosidic aroma precursors" en la 2th International Conference on Food Oral Processing, Beaune, France, 1-5 June, 2012. **Carolina Muñoz-González**, Juan J. Rodríguez-Bencomo, Carolina Cueva, Serkan Selli, M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón.

- Este trabajo titulado "Capacidad de la microbiota oral humana para producir compuestos odorantes del vino a partir de precursores no

odorantes de la uva" va a ser presentado en el XIX Congreso Nacional de Microbiología de los Alimentos, Zaragoza, España, 24-26 Sept, 2014. **Carolina Muñoz-González**, Carolina Cueva, M. Ángeles Pozo-Bayón, M. Victoria Moreno-Arribas.

Publicación 7. Recuperación de agliconas aromáticas a partir de subproductos de vinificación mediante extracción líquido-líquido y extracción con líquidos presurizados.

Recovery of aromatic aglycones from grape pomace winemaking by-products by using liquid-liquid and pressurized-liquid extraction

Carolina Muñoz-González; Juan J. Rodríguez-Bencomo; Pedro J. Martín-Álvarez; M. Victoria Moreno-Arribas, M. Ángeles Pozo-Bayón.

Food Analytical Methods, 7 (1) 2014, 47-57

Abstract

The potential of winemaking grape pomace by-products as a source of glycosidic aroma precursors that under enzymatic hydrolysis might release aroma compounds has been evaluated. Two different extraction methodologies, liquid-liquid and pressurized-liquid extraction (LLE and PLE) were employed. Solid phase extraction (SPE)-GC-MS analysis of the hydrolyzed LLE glycosidic extract revealed 22 aroma compounds belonging to different chemical families (terpenes, C13 norisoprenoids, vanillines, etc.). Response surface methodology was employed to study the effect of the most significant PLE experimental variables (temperature and solvent composition) on the extraction of aromatic aglycones. The parameters of the model were estimated by multiple linear regressions. Most of the aroma compounds showed an adequate fit to the calculated model (18 compounds from 22 with $R2>0.8$). The application of the optimized PLE conditions (50% of ethanol in the hydroalcoholic solution) and 90 ºC showed higher extraction yield of aglycones when comparing with the extraction yield obtained by LLE.

Introduction

Grape pomace consists in the skin, stems and seeds of grapes that remain after processing in the wine and juice industry. Recently, it has been stated that 10 million tons of grape pomace was produced in 2005 from 66 million tons of harvested grapes (Vitis vinifera L.) in the world (Maier et al., 2008). Grape processing wastes can be an important economical problem to producers besides the environmental impact caused by the large amount of these types of residues generated during the harvest season. The majority of this pomace is discarded as natural waste or distilled to produce alcohol and other distilled beverages. However, as Fernández and collaborators have recently pointed (Fernández et al., 2010) the new regulation in the reform of the Common Organization Market (OCM) of wine eliminates the subsidy to distillation in 2013. Therefore, the wineries will have new economic difficulties with winery waste management.

Besides some traditional applications of grape pomace for animal fed formulations (Brenes et al., 2008) or compost production (Bustamante et al., 2008) in the latest years, the scientific works carried out on the characterization of the chemical components of waste grape by-products has allowed looking for different applications in trying to obtain high added value ingredients. Some of these applications are the production of grape seed oil (Fiori 2007) or biodiesel from it (Fernández et al., 2010), obtaining dietary fibre (Igartuburu et al., 1998; Pérez-Jiménez and Sáyago-Ayerdi, 2009; González-Centeno et al., 2010) and mainly in the last years, the extraction of polyphenols with antioxidant properties (Louli et al., 2004; Chafer et al., 2005; Guendez et al., 2005; Pinelo et al., 2005; Hogan et al., 2010; Monrad et al., 2010) for food, cosmetic or pharmaceutical applications.Some other potential applications, such as the use of grape pomace to recover aroma compounds have been less explored. Ruberto and collaborators (2008) explored this possibility, but they focused on the free volatile profile of grape pomace coming from the processing of different grapes varieties (Nero d'Avola, Nerello Mascalese, Frappato and Cabernet Sauvignon), showing a volatile profile mainly dominated by carboxylic acid derivatives with relatively high odor thresholds.

However, grape aroma compounds can be present both as free volatiles and in much higher concentrations, as non-volatile sugar-bound glycoside conjugates (Baumes, 2009). The occurrence of glycosidically bound volatiles is typically two to eight times greater that of their free counterparts (Maicas and Mateo, 2005) and, although their distribution in the grape berry might change during ripeness (Park et al., 1991) they are present in the largest amount in the skin (Gomez et al. 1994). In spite of the fact that grape glycosides are non-volatile odorless flavor precursors, under enzymatic or acid hydrolysis during winemaking they can release the corresponding odorant aglycones, which are generally potent flavor compounds (monoterpenes, norisoprenoids, benzenoids compounds, etc) characterised by low aroma thresholds and interesting sensory properties (Maicas and Mateo, 2005).

In spite of the evident interest of using grape glycosides as a source of aroma compounds, the works focused on the characterization of glycosides in grape pomace are scarce in the literature. Only Vasserot and collaborators (1993) carried out pioneer studies, in which they evidenced the presence of monoterpenol glucosides in Muscat grape by-products. Nonetheless, in their study, the characterization of the released odorant aglycones, which are the interesting compounds as a source of natural flavors, was not performed, since they quantified the total amount of monoterpenols using a colorimetric assay.

For the extraction of grape aroma glycosides, most of the works in the literature use liquid- liquid extraction employing hydroalcoholic solutions letting the sample macerate in the darkness during long extraction times (24 h at least) (Hernández-Orte et al., 2008; Hernandez-Orte et al., 2009; Loscos et al., 2010). However, other technologies, such as the use of supercritical CO_2 extraction has also been successfully used (Palma et al., 2000), although in the above-mentioned work the characterization of the corresponding odorant aglycones was not performed. The use of pressurized liquid extraction (PLE) is a relatively new extraction approach that is being applied for the extraction of different types of phytochemicals from plants (Lou et al., 1997; Ju and Howard, 2003; Pinelo et al., 2005; Monrad et al., 2010). The use of high pressure-high temperature extraction might increases the contact with the solvent facilitating solvent penetration into complex matrices such as grape pomace. In addition, other advantages are the relatively short extraction times and the possibility of using GRAS solvents or even water (subcritical water), which makes PLE a “green” extraction methodology

(Richter et al., 1996; Lou et al., 1997). Different procedures using PLE have been optimized for the extraction of some phytochemicals from grape pomaces (Ju and Howard, 2003; Monrad et al., 2010) in recent years. However, as far we now, none scientific work has evaluated the use of PLE for the extraction of glycoside aroma precursors.

Therefore, the objective of this work has been firstly to check the potential of grape pomace (Verdejo white grape variety) as a source of glycosides that under enzymatic hydrolysis might release aroma compounds, and secondly, to know the feasibility of PLE for the extraction of these glycosides comparing it with the more conventional liquid-liquid extraction (LLE).

Materials and Methods

Grape Pomace Samples

Grape pomace from Verdejo white grape variety was provided by a winery from the O.D Rueda (Spain). Fresh pomace from pressed grapes (pneumatic pressing) previously submitted to a maceration process (without fermentation), was immediately recovery, and placed into plastic bags in absence of oxygen, sealed and stored at -20 ºC. Frozen grape pomace was dried in a lyophiliser (Labonco, Kansas City, MO, USA) and ground into a fine and homogenous powder using a commercial coffee grinder. The powder was stored at -20 ºC in absence of oxygen till it was used for the analyses.

Extraction of glycosidic aroma precursors from grape pomace by liquid-liquid extraction (LLE)

The procedure for the extraction of aroma precursors from grape pomace was based on that described by Hernandez-Orte and co-authors (2009) (Hernandez-Orte et al., 2009) with some modifications. One hundred grams of the grape pomace powder were suspended in 500 mL of a buffer solution (0.1 M Na2HPO4/NaH2PO4) at pH 7 and 13% (v/v) ethanol (Scharlau Chemie S A., Barcelona, Spain) allowing macerating in the darkness in absence of oxygen (60 h, 20 ºC in a nitrogen atmosphere). This solution was centrifuged at 16770 x g for 15 min at 20 ºC, and the supernatant was filtered through filter paper. Ethanol was removed from the sample by using a Rotavapor R-200 (Buchi Labortechnik AG, Flawil, Switzerland) at 25 ºC.

Extraction of glycosidic aroma precursors from grape pomace by pressurized-liquid extraction (PLE)

Aroma precursors were extracted from the grape pomace by using an accelerated solvent extractor (ASE 200, Dionex Corporation, Sunyvale, CA) equipped with a solvent flow controller. Two solvents of different polarity, ethanol (Scharlau Chemie S.A.), and purified water by using a Milli-Q system (Millipore, Bedford, MA) were employed. Freeze-dried grape pomace (9 g) was dispersed thoroughly with 9 g of sea sand (Panreac, Barcelona, Spain). The homogeneous mixture was loaded into a 33-ml extraction cell with a cellulose paper filter at the bottom of the cell. PLE experimental variables were pressure (1500 lb/in2), three extraction cycles, flush volume (60 %), nitrogen purge time (60 s), static time (8 min) and preheat time (5 min). Ethanol was removed from the collected sample by using a Rotavapor R-200 (Buchi Labortechnik AG, Flawil, Switzerland) at 25 ºC. The experiment was repeated until the complete extraction of 50 g of grape pomace.

Solvent and temperature optimization in the PLE method

The effect of two factors, solvent type (S) and temperature (T), on the relative peak area of each aroma compound (response variable) obtained after the hydrolysis of the grape glycoside aroma precursors recovered from the grape pomace was evaluated by using a central composite circumscribed (CCC) design (Box et al., 1978). A total of 10 assays: four points of a full factorial design (combination of levels -1 and +1), four star points (at levels ± α, α = start distance = 1.414), and two centre points to estimate the experimental error, were carried out in randomized run order. By using this design, the two factors were tested at five different experimental levels: the concentration of ethanol employed in the hydroalcoholic mixture as solvent (S) at 0, 15, 50, 85 and 100 (% v/v EtOH); and the temperature (T) at 48, 60, 90, 120 and 132 (ºC); in correspondence with the coded levels: -1.414, -1.000, 0, +1.000, +1.414, respectively. **Table 1** shows the experimental matrix design, with the experimental levels of the independent variables (factors).

Table 1. Experimental matrix design for the PLE factors: percentage of ethanol in the hydroalcoholic solution (S) and temperature (T).

Nº Assay	*S* (%)	*T* (ºC)
1	0	90
2	50	90
3	15	60
4	85	60
5	85	120
6	100	90
7	50	48
8	50	132
9	15	120
10	50	90

The quadratic polynomial model proposed for the response variable ( ) for each selected volatile compound was:

$$Y_i = \beta_o + \beta_1 S + \beta_2 T + \beta_{1,1} S*S + \beta_{2,2} T*T + \beta_{1,2} S*T + \varepsilon \quad \text{(Equation 1)}$$

Where is the intercept, the linear coefficients, the quadratic coefficients, the interaction coefficient, and is the variable error. The parameters of this model were estimated by multiple linear regression (MLR) using the Statgraphics Centurion XV program (StatPoint Inc., www.statgraphics.com) that permits the creation and analysis of experimental designs. The effect of each term and their statistical significance for each of the response variables (aroma compounds released from the corresponding glycosides) were analysed from the standardized Pareto chart. The goodness of fit of the model was evaluated by the coefficient of determination (R2) and the residual standard deviation (RSD). The terms not significantly different from zero ($p > 0.10$), were excluded of the model and the mathematical model was re-fitted by MLR. From the fitted model, the estimated surface plot and the optimum conditions that maximized the response variable were obtained.

Isolation of glycosides aroma precursors from the grape pomace extracts by using solid phase extraction (SPE)

The glycosides aroma precursors contained in the extracts obtained by LLE or PLE were isolated by adsorption onto an Amberlite XAD-2 (Supelco, Bellefonte, USA), column. A 10 cm length glass column (Pobel, Madrid, Spain), filled with 40 g of

Amberlite XAD-2 was prepared by sequentially conditioning it with 120 mL of dichloromethane, methanol and water. The sample extract was introduced into the column which was afterward rinsed it with 100 ml of water and 150 ml of pentane/dichloromethane (2:1 v/v) to remove any residual of free volatiles. Elution of the glycosides aroma precursors was performed with 150 mL of ethyl acetate/methanol (9:1 v:v). This fraction was collected and solvent was evaporated by using a rotavapor (Buchi Labortechnik AG). The dried extract was reconstituted in 4 mL of water, extracted twice with 1 mL of dichloromethane and 1 ml of pentane to ensure the complete removal of free volatiles, aliquoted and stored at -20ºC. The absence of free volatiles in the aroma precursor extract was further tested.

Release of aromatic aglycones from the glycosidic extracts by enzymatic hydrolysis

Previous to the GC-MS analysis, the glycoside extracts from grape pomace were submitted to enzymatic hydrolysis to release the corresponding free aroma compounds (aglycones). Enovin® (Agrovin, Ciudad Real, Spain), a commercial oenological enzymatic preparation of several Aspergillus niger (GMO free) with β-glucosidase activity was used to release the odorant aglycones. The enzymatic preparation was dissolved in a citrate/phosphate buffer (pH=5; 51.5% 0.2 M sodium phosphate and 48.5% 0.1 M citric acid) and 500 µL of a 20 mg/mL of the enzyme solution were added to the glycosidic precursors extract. The amount of enzyme was previously optimised to provide the maximum hydrolysis yield. After the addition of 50 µL of a 90 mg/mL solution of n-octylglucoside in ethanol as internal standard, the mixture contained in a tube was closed and placed in a bath at 40 º C for 16 h. The hydrolyzed was cooled over ice, and the released aglycones were analysed by SPE following the procedure described as following.

Analysis of the aroma compounds released from the glycosidic aroma precursors by SPE-GCMS

The SPE was carried out using the method proposed and validated by Loscos and collaborators (2010) with slight modifications. The total volume of the glycoside hydrolisate containing 20 µL of a solution of β-damascone from Sigma-Aldrich (0.25 mg/ml in ethanol) as internal standard (previously, it was checked its absence in the hydrolysed extract) was passed through a 50-mg LiChrolut EN cartridge (Merck KGaA, Darmstadt, Germany) previously pre-conditioned (2 mL of dichloromethane, 2 mL of

methanol and 2 mL of a 12% ethanol solution). The sorbent was washed with 5 mL of 40 % (v/v) methanol solution and dried by letting air pass through (0.6 bar, 10 min). Aglycones were recovered by elution with 1 mL of dichloromethane. Twenty microliters of an internal standard solution (4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone and 2-octanol at a concentration of 465.5, 598.5 and 665 µg in 10 ml of dichloromethane) were added to the eluted sample. The extract was concentrated under a gentle stream of nitrogen to a final volume of 100 µL and then analyzed by GC-MS under the conditions described below.

Two microliters of the aroma extracts were directly injected in splitless mode into the GC-MS (Agilent 6890) provided with an Agilent MSD ChemStation software to control the system. For separation, a Supra-Wax fused silica capillary column (60 m × 0.25mm i.d. × 0.50 µm film thickness) from Konik (Barcelona, Spain) preceded by a 50 cm x 0.25 mm uncoated and deactivated precolumn from Quadrex (Woodbridge, CT, USA) was used. Helium was the carrier gas at a flow rate of 1 mL/min. The oven temperature was initially held at 40 ºC for 5 min, then increased at 4 ºC/min to 240 ºC and held for 20 min.

For the MS system (Agilent 5973N), the temperatures of the transfer line, quadrupole and ion source were 270, 150 and 230 ºC respectively. Electron impact mass spectra were recorded at 70 eV ionization voltages and the ionization current was 10 µA. The acquisitions were performed in Scan (from 35 to 350 amu) and SIM modes for some specific compounds. The signal corresponding to a specific ion of quantification was calculated by the data system. The identification of compounds was carried out by comparison of retention times and mass spectra of the references compounds with those reported in the mass spectrum library NIST 2.0. Quantitative data were obtained by calculating the relative peak area in relation to that of the corresponding internal standard. To calculate the concentration of each aroma compound, calibration curves of each reference compound at different concentrations covering the concentration ranges expected in the samples were prepared in dichloromethane and analysed in the same conditions that the samples. To do so, standards of volatile compounds of the maximum purity available (>98%) were purchased from different providers: Aldrich (Steinheim, Germany); Fluka (Buchs, Switzerland); Merck (Munchen, Germany) and Firmenich (Geneve, Switzerland). These compounds are shown in **Table 2**.

Table 2. Aroma compounds identified in the glycoside extract from grape pomace after the hydrolysis using LLE and PLE and comparison (in % extraction yield) between both procedures.

Nº [a]	Aroma compounds	RT[b]	Ion Q[c]	ID[d]	LLE (mean ± SD) (µg/kg dry pomace)	PLE (mean±SD) (µg /kg dry pomace)	LLE *vs* PLE (%)[e]
1	Limonene	21.63	68	1	0.85±0.19	4.33±1.12	19.57
6	Linalool oxide	31.42	59	1,2	1.09±0.09	2.48±0.17	43.82
9	Nerol	41.28	69	1	1.18±0.05	Traces	-
10	Geraniol	42.50	123	1	5.52±0.8	12.52±4.86	44.1
17	8-Hydroxylinalool	53.91	121	1,2	Traces	6.55±2.33	-
	Sum				*9.45*	25.90	
	C13 Norisoprenoids						
21	Oxo-alpha-ionol	63.12	108	1,2	28.61±2.78	53.77±37.22	53.2
	Volatile phenols						
14	Eugenol	51.13	164	1	1.24±0.02	3.37±0.02	36.71
15	2-Methoxy-4-vinylphenol	51.83	135	1	176.77±14.21	1872.9±453.9	9.44
16	2,6-Dimethoxyphenol	53.61	139	1	1.41±0.43	Traces	-
18	4-Vinylphenol	56.15	120	1	Traces	590.8±183.03	-
	Sum				*179.42*	*2467.13*	
	Vanillins						
19	Vanillin	61.26	151	1	1.32±0.27	114.0±46.34	1.15
20	Methyl vanillate	62.30	151	1	2.94±0.01	9.01±0.1	32.66
22	Acetovanillone	63.63	166	1	9.77±1.60	41.55±5.36	23.51
	Sum				*14.02*	*164.56*	
	Benzenoids						
7	Benzaldehyde	33.63	106	1,2	93.69±9.74	378.35±26.19	24.76
11	Benzyl alcohol	43.68	79	1	241.65±51.84	443.28±57.91	54.51
12	*β*- Phenylethyl alcohol	44.67	91	1	136.05±21.34	380.10±16.71	35.79
	Sum				*471.40*	*1201.74*	
	Lipids derivatives						
2	1-Hexanol	27.02	56	1	79.31±6.56	278.25±10.55	28.5
3	*trans*-3-hexen-1-ol	27.44	67	1	1.26±0.16	3.81±0.40	32.94
4	*cis*-3-hexen-1-ol	28.22	67	1	4.56±0.37	13.58±0.91	33.58
5	*trans*-2-hexen-1-ol	28.92	57	1,2	22.75±0.25	80.86±4.91	28.13
8	2-Octen,1-ol	35.78	57	1,2	1.23±0.02	5.12±0.62	24.01
13	γ-Nonalactone	48.05	85	1	1.61±0.26	22.80±2.64	7.08
	Sum				*110.72*	*121.07*	

[a] Peak number in the chromatogram depicted in **Figure 1**; [b] RT: Retention time (min), [c] IonQ : quantification ion; [d] ID: identification based on mass spectra using reference compounds (1) or comparing their mass spectra with those included in the NIST library (2). [e] For the comparison between LLE and PLE, results from PLE extraction were considered as 100% extraction. *N=2* different samples submitted to the complete extraction-hydrolysis-volatile analysis procedure.

Results and Discussion

Aroma compounds released after the hydrolysis of aroma precursor glycosides recovered from grape pomace using LLE

Aroma compounds were released from the aroma glycosides extracted from Verdejo grape pomace by using commercial fungal glycosidases, therefore in trying to obtain a more natural flavour profile compared to the acidic hydrolysis, which has been indicated it might induce a molecular rearrangement and the transformation of some of the liberated aglycones (Maicas and Mateo 2005). The aroma composition after the enzymatic hydrolysis of the glycoside extracts recovered by LLE was shown in Table 2. A total of 22 varietal aroma compounds belonging to different chemical families (terpenes, C13 norisoprenoids volatiles phenols, benzenoids, vanillines and lipid derivatives) were identified based on their characteristic gas chromatography and mass spectra data.

Figure 1 shows a chromatogram of the typical GC-MS volatile profile of the varietal compounds released from the glycosidic aroma precursors.

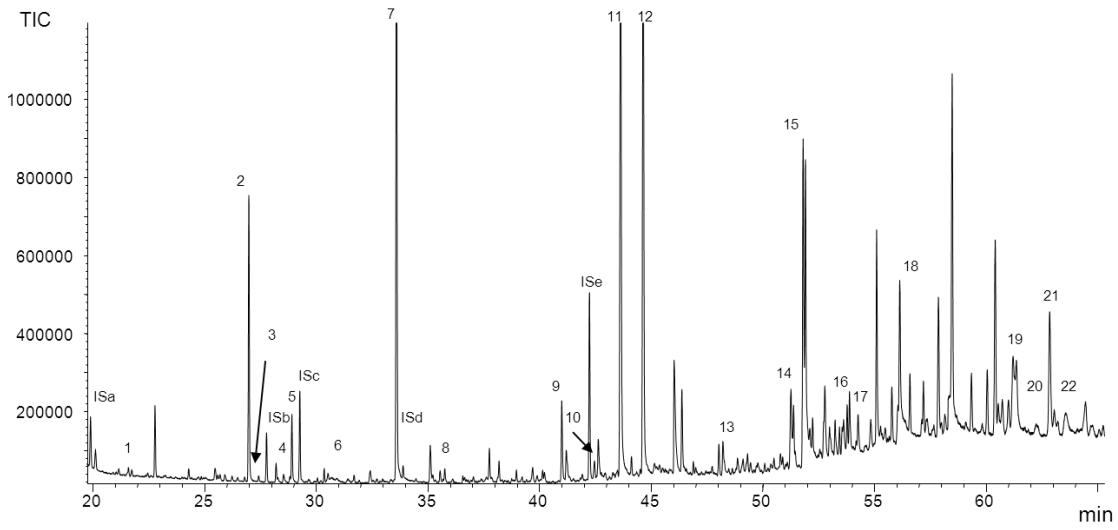


Figure 1. Chromatogram corresponding to the aroma compounds released after the enzymatic hydrolysis of the aroma precursors glycosides extracted by LLE from grape pomace. Peak identities are shown in Table 2. ISa, ISb, ISc, ISd and ISe correspond to the internal standards 4-methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanol, 2-octanol, 1-octanol and β-damascone.

For instance, the monoterpenes limonene, nerol, geraniol and two linalool related compounds such as 8-hydroxylinalool and linalool oxide were detected in the

grape pomace in an average concentration of 10 µg of monoterpenes / kg dry pomace, in which geraniol, was the monoterpene extracted at the highest amount. In wines, this compound presents a floral aroma. Moreover, geraniol and linalool are compounds associated to the pleasant Muscat like odor (Etievant 1991). Many monoterpenoids have been associated to pleasant floral aroma attributes and it is important to notice that in general, they present very low odor thresholds (100-400 µg/L) (Baumes 2009). Another poly-oxygenated terpene identified in the pomace extract was the compound 8-hydroxylinalool. Although by their own, poly-oxygenated terpenes might have small sensory relevance, they can be transformed into odorant monoterpenols by hydrolysis at acid pH (Strauss et al., 1987). Linalool, one of the most common odorant aglycones released from some floral grape varieties such as Muscat, Riesling and Gewürztraminer, was absent in the hydrolyzed grape pomace extract, which might be due to its oxidation via the formation of an epoxide into different types of linalool oxides. In fact, linalool oxide was also identified in the LLE extract (table 2 and peak nº 6 in Figure 1). The presence of other types of hydroxylated linalool derivatives has been described in the bound fraction of other white grape varieties such as Muscat and Melon B grape varieties (Sánchez Palomo et al., 2006). The compound oxo-α-ionol was the only C13 norisoprenoid identified in the hydrolyzed extract. However, it was one of the quantitatively most abundant compounds extracted from the grape pomace (28.6 µg / kg dry pomace). This compound has been associated with a spicy aromatic note and as opposite to terpenes, it is normally found in the same quantities in all the grape varieties, aromatics or neutral (Maicas and Mateo 2005). Table 2, also shows the four volatile phenols and three related compounds (vanillins) that were identified in the pomace extract. In wines, these compounds might contribute to wine flavor because of their low odor thresholds. Their presence in the pomace extract is likely due to the hydrolysis of the corresponding glycosidic precursors (Strauss et al. 1987). However, some vanillins could have been formed from ethanolysis of lignin (Etievant 1991), which forms part of the stem and seeds present in the pomace, and the use of ethanol employed as extracting solvent during the extraction. Among the volatile phenols, the compound 2-methoxy-4-vinylphenol, was present at the highest amount (176.76 µg/kg dry pomace). This compound exhibits a very low odor threshold (10 µg/L in water) (Etievant 1991), and it has been related to clove-like, balsamic, peppery-woody aroma nuances (Campo et al., 2005). Among the vanillins, acetovanillone, was the quantitatively most important compound detected in the pomace extract (9.77 µg/kg

pomace extract). The three vanillins identified in the extracts (vanillin, methyl vanillate, acetovanillone) have been associated with pleasant vanilla aromatic notes in wines (Aznar et al., 2001; Escudero et al., 2002). In addition, three benzenoids compounds (benzyl alcohol, β-phenylethyl alcohol and benzaldehyde) were also identified. Taking into account their interest for their aroma characteristics, β-phenylethyl alcohol could be the most interesting one, which has been related to rose-like odor. This compound was detected in the extract in a relatively large amount compared to other aglycones (above 136 µg/kg dry pomace) (Table 2). The amount of this compound in the pomace extract was even higher than that reported by Gómez and co-authors (Gomez et al., 1994) in the skin of other non-aromatic grape varieties, such as Monastrell, Cabernet Sauvignon and Tempranillo (43, 72 and 73 µg / kg grape respectively). Although the origin of β-phenylethyl alcohol in many fermented beverages is from the catabolism of amino acids during the alcoholic fermentation, this compound can occur in the fruit berry (e.g grape) in a rather high concentration as a non-volatile precursor bound to an uncharacterised glycoside residue (Wilson et al. 1984). Table 2 also shows some lipids derivatives identified in the pomace extract corresponding, in general, to some C6 aliphatic alcohols and the lactone γ-nonalactone. It has been shown, that some C6 aliphatic alcohols might be in the grape as odorless β-D-glycosides (Sánchez Palomo et al., 2006). In fact, it has been reported that while in aromatic grapes monoterpenols are important aglycones, in the case of non-aromatic grapes, instead of monoterpenols, the C6 aliphatic alcohols are the most preponderant varietal alcohols (Gomez et al. 1994). Most of them are associated to green-herbal aroma nuances (Hashizume and Samuta, 1997; Ugliano and Henschke, 2009). The only lactone identified in the extracts, was γ-nonalactone, although its concentration was relatively low (1.6 µg / kg dry pomace). Nonetheless, it could be interesting because of its aroma characteristics, since it has been shown it possess a lower odor threshold (30 µg/L) and a pleasant odor described such as coconut-like (Escudero et al., 2007).

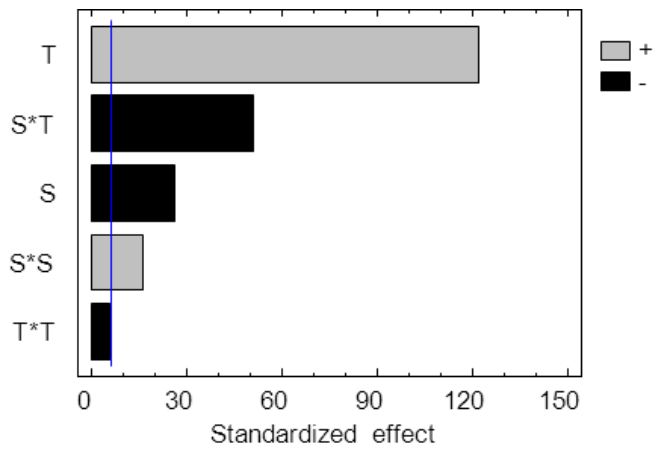
Therefore, the hydrolyzed extract from Verdejo grape pomace showed different types of varietal aroma compounds mainly characterised by very low detection thresholds and many of them associated to pleasant aromatic notes. Taking into consideration their aroma characteristics, this aroma extract seems more interesting for different types of industrial applications, than the remaining free volatiles fraction

present in the grape pomace previously considered for the valorisation of this type of wine by-products (Ruberto et al., 2008).

Optimization of a procedure based on PLE for the recovery of aroma precursor glycosides from grape pomace

Once it was proven that grape pomace contained glycosides that after hydrolysis can release a wide spectrum of aroma compounds, the next step in the work was looking for an extraction method allowing the maximum glycoside extraction yield. To do so, PLE was chosen for this objective. This technique has been recently and successfully used for the recovery of other grape phytochemicals from red grape pomaces (Ju and Howard, 2003; Monrad et al., 2010). For the optimization of the best extraction conditions, we focused on the effect of the extracting solvent (different hydroalcoholic solutions) and temperature, since they are outstanding variables in the PLE extraction procedure (Richter et al., 1996; Lou et al., 1997). The relative peak areas of the aromatic aglycones released after the hydrolysis of the extracts obtained by PLE were calculated in the different analysis conditions provided by the experimental matrix of the factorial design (Table 1). These ranges were chosen on the basis of previous works based on the extraction of other grape phytochemicals (Ju and Howard, 2003; Monrad et al., 2010). All the experiments were randomly performed to minimize the effect of uncontrolled factors that might introduce bias in the measurements. MLR was applied to estimate the parameters of the proposed model in Equation 1 for all the aglycones identified in the extracts (response variables). The effect of each parameter in the model and their statistical significance were analyzed from the Pareto chart. Figure 2a shows an example, in which the effect of each term of the model divided by its standard error is shown.

a)



b)

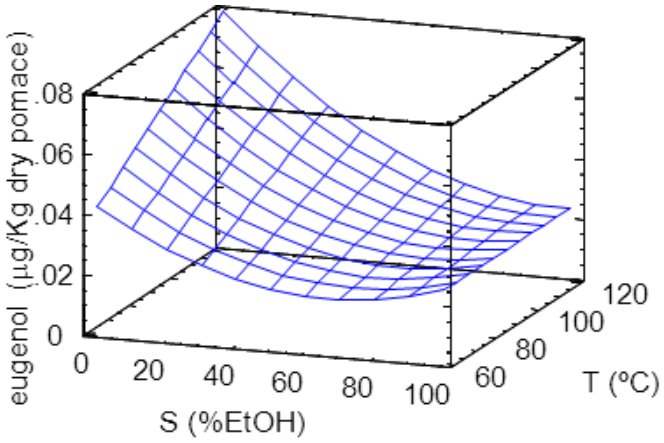


Figure 2. a) Standardized Pareto Chart plot with the effect of each term the model divided by its standard error for the response variable eugenol (µg/kg dry pomace). The vertical line tests the significance of the effects at the 90% confidence level. Legend for the bars corresponds to the terms in the model of Equation 1. **b)** Surface plot of the estimated response variable (eugenol, g/kg dry pomace) as a function of the extraction temperature, T(ºC) and solvent, S (% of ethanol in the hydroalcoholic mixture).

The terms not significantly different from 0 ($p < 0.10$) were excluded of the model and the mathematical model was refitted. The regression coefficients, for unscaled factors and the statistics of the fitting for each response variable (determination coefficient, and residual standard deviation RSD) are also shown in **Table 3**. As can be seen, most of the aroma compounds released from the pomace glycosides, showed an adequate fit to the calculated model (18 compounds from 22 with $R^2 > 0.8$). Only four compounds, 2-methoxy-4-vinyl-phenol, 8-hydroxylinalool, oxo-α-ionol and 4-vinylphenol showed an inadequate fit to the proposed model. In the table, it can be seen that the linear terms with the strongest influence on the recovery of odorant aglycones after the hydrolysis were both the extracting solvent composition (S) and the temperature (T) having in general, a negative and a positive influence, respectively. Only four compounds were negatively affected by the temperature: limonene, γ-nonalactone, 8-hydroxylinalool and oxo-α-ionol, although the two latter ones also showed inadequate fits to the model. It seemed clear that solvent composition (% of ethanol/water) affected the glycoside extraction from grape pomace as has been also shown for the extraction of other grape phytochemicals (Pinelo et al., 2005; Luque-Rodríguez et al., 2007; Makris et al., 2008). Considering the temperature, the significant effect of this factor during the PLE extraction, might be explained because it provokes an increase in mass transfer favoring the solubility of the metabolites of interest (Richter et al., 1996; Lou et al., 1997). The quadratic terms (S2 and T2) seemed to be less

important for the model, although T2 showed a significant and negative effect for many compounds, confirming the large effect of temperature in the extraction. On the contrary, the interaction term (S x T) did not seem very significant, and only five compounds (γ-nonalactone, 2,6-dimethoxyphenol, methyl vanillate and 4-vinylphenol) were affected.

When comparing the optimum values (maximum values of relative peak area) for the extraction of each aroma compounds, there were not an ideal solvent/temperature conditions valid for all of them likely due to the structural differences and complexity of the different types of glycosides present in the grape pomace (Wilson et al., 1984; Maicas and Mateo, 2005). This has been already stated when optimizing the extraction conditions of other structurally complex grape phytochemicals such as anthocyanins (Ju and Howard, 2003; Monrad et al., 2010).

In addition, some of the extraction conditions essayed, specifically those not involving the use of ethanol (extraction with subcritical water), gave a lot of operational and technical issues during the extraction procedure (clogging valves and tubes of the ASE device), possibly because of the extraction of other polar compounds from the grape pomace (peptides, proteins, pectines, polyphenols) that made unviable the use of low ethanol hydroalcohlic mixtures. Therefore, the optimal extraction conditions were chosen taking into consideration those which provided the highest extractions (µg/kg grape pomace) of the majority of aromatic aglycones, which were obtained during the assay number 2 and 10 (**Table 1**) using 50% of ethanol in the hydroalcoholic solution and 90 ºC as extraction temperature. Figure 2b shows an example of the surface plot for the optimal extraction conditions calculated for one of the aromatic aglycones. In this case, as can be shown, although the best extraction yield was obtained at lower ethanol concentration, as it was stated before, compromise conditions were used in order to obtain higher extraction yield, but avoiding technical and operational problems in the extractor device.

Therefore, the optimized PLE conditions (50 % ethanol and 90 ºC) were applied for the extraction of glycosidic aroma precursors from grape pomace. The compounds identified and their concentrations after the enzymatic hydrolysis are also shown in Table 2. As can be seen in the table, these data were compared to those previously obtained by using LLE (ethanol 13% v/v at room temperature during 60 hours in the

darkness). Compared to the most conventional extraction procedure (LLE), the extraction efficacy of the PLE was higher. The hydrolyzed extracts obtained by PLE had considerably higher amounts of the majority of varietal aglycones whatever the chemical family considered. Only the amounts of lipid derivatives were more or less similar indistinctly of the extraction method used. It is worth to notice that almost 50% more terpenes derivatives were found in the PLE extracts. However, nerol was not detected in this extract, which might be due to a minor conversion rate from its precursor, geraniol (Park et al., 1991), because of the shorter extraction time applied during the PLE procedure compared to the LLE method. The compound 2,6-dimethoxyphenol was not identified in the PLE extract either, although its concentration was also very low by using LLE (Table 2). On the contrary, the three other volatile phenols, eugenol and mainly 2-methoxy-4-vinylphenol and 4-vinylphenol, were higher extracted by using PLE (3.7, 1872.9 and 590.8 µg/kg dry pomace respectively). In addition, very important differences between both extraction methods were observed in the extraction of vanillines, and for example, vanilline was above 90 % more extracted using PLE than LLE (only about 10% extracted using LLE) (Table 2). Benzenoids compounds were only between 24 and 54% extracted using LLE compared to the PLE. These results showed that PLE was more effective in the extraction of glycosides from grape pomace than the more conventional LLE method. This higher effectiveness can be linked to the advantages associated of using an ethanolic mixture at high pressure and high-temperature compared to a conventional method also using a hydroalcoholic mixture but in static conditions during longer extraction times. However, it is important to consider, than in spite of the higher extraction rate of glycosides (therefore, of the corresponding aromatic aglycones) associated to the PLE method, some drawbacks of this procedure have also been noticed during this work. First of all, the limited amount of sample that can fit in the extraction cell (using a conventional ASE device), which makes necessary many repeated extraction cycles, and secondly, some operational problems when using higher proportion of water in the hydroalcoholic solution, which could be of interest lowering the solvent cost and making possible the use of more environmental friendly solvents, because of the high extraction of other grape-polar compounds.

Table 3. Estimated Regression coefficients for unscaled factors in Equation 1 and statistics for the fit obtained by MLR for the aroma compounds released from the glycosides extracted from the grape pomace by using PLE after the hydrolysis.

	Terms in the model						**Statistics for goodness of fit**	
Variables	***Constant***	***S***	***T***	***S^2***	***T^2***	***S x T***	***R^2***	***RSD***
Limonene	0.00635803	0.00000615403	-0.000159511		0.00000125153		0.979	0.0005
1-Hexanol	4.84129	-0.154709	0.0653037	0.00101289	-0.000372364		0.990	0.0762
Trans-3-Hexen-1-ol	0.0446179	-0.00169028	0.00112477	0.0000106278	-0.0000067112		0.983	0.0016
Cis-3-Hexen-1-ol	0.148667	-0.00559274	0.00437546	0.0000354289	-0.0000266963		0.992	0.0043
Nerol (cis-geraniol)	-0.0396537	-0.000159837	0.00195348		-0.00001190		0.923	0.0043
Geraniol	-0.0112303	-0.00011636	0.000850225		-0.0000049728		0.870	0.0023
Benzyl alcohol	0.295502	-0.0142828	0.137788		-0.000905724		0.818	0.6859
β-Phenylethyl alcohol	-2.1666	-0.0267202	0.25123		-0.00146442		0.960	0.3509
γ-Nonalactone	0.325613	-0.00343864	-0.00197857			0.0000289093	0.931	0.0092
Eugenol	0.00551019	-0.00034925	0.000615012	0.00000546219		-0.0000062388	0.998	0.0006
2-methoxy-4-vinylphenol	0.914947	-0.00269368	0.00357283				0.448	0.1654
2,6-dimethoxyphenol	-0.0386397	0.00036808	0.00109878		-0.00000548	-0.0000044413	0.992	0.0010
Vanillin	0.0833462	-0.00864883	0.0107109				0.948	0.1061
Methyl vanillate	0.529375	-0.030944	0.0248154	0.000244053	-0.000121143	-0.0000870422	0.999	0.0026
Acetovanillone	-0.318261	-0.0037661	0.026272		-0.000154543		0.970	0.0337
Benzaldehyde	10.032	-0.449241	0.335528	0.00286369	-0.00195865		0.982	0.4449
8-Hydroxylinalool	0.0230286	-0.000161169	0.000072984				0.442	0.0057
Trans-2-hexen-1-ol	0.78253	-0.0288612	0.0191629	0.000181485	-0.000113484		0.991	0.0200
Linalool oxide	-0.0000131711	-0.0000404389	0.000209486		-0.000001191		0.818	0.0007
Oxo-α-ionol	0.204589	0.0002608	-0.000851469				0.372	0.0414
2-Octen,1-ol	-0.0585435	-0.00041849	0.00366138		-0.0000208908		0.972	0.0041
4-Vinylphenol	-0.852878	0.0192358	0.008343			-0.000245096	0.649	0.2256

S % ethanol in the hydroalcoholic mixture, *T* extraction Temperature, R^2 determination coefficient, *RSD* residual standard deviation

Conclusions

The results of this work show that grape pomace by-products can be a source of glycosidic aroma precursors that after hydrolysis can release interesting odorant compounds based on their aroma quality and low odor thresholds. The use of PLE working in the optimised conditions (50% ethanol/water, 90 ºC) greatly improves the extraction compared to the more conventional LLE. Considering the large amount of grape pomace produced every year in the world, the extraction of aroma glycosides can be an interesting alternative for the recovery and valorisation of grape by-products with potential applications in different industrial sectors (agro-food, cosmetic, perfumery, etc.) besides reducing their environmental consequences.

References

Aznar M, López R, Cacho JF, Ferreira V (2001). Identification and quantification of impact odorants of aged red wines from Rioja, GC-olfactometry, quantitative GC-MS, and odor evaluation of HPLC fractions. J. Agric. Food Chem. 49(6): 2924-2929

Baumes R (2009). Wine aroma precursors. In: M. V. Moreno-Arribas and M. C. Polo (eds). Wine Chemistry and Biochemistry. Springer, New York, pp 251-274

Box GEP, Hunter WG, Hunter JS (1978). Statistics for experimenters An introduction to design, data analysis, and model building. New York John Wiley & Sons

Brenes A, Viveros A, Goñi I, Centeno C, Sáyago-Ayerdy SG, Arija I, Saura-Calixto F (2008). Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. Poultry Sci. 87(2): 307-316

Bustamante MA, Moral R, Paredes C, Pérez-Espinosa A, Moreno-Caselles J, Pérez-Murcia MD (2008). Agrochemical characterisation of the solid by-products and residues from the winery and distillery industry. Waste Manage. 28(2): 372-380

Campo E, Ferreira V, Escudero A, Cacho J (2005). Prediction of the wine sensory properties related to grape variety from dynamic-headspace gas chromatography-olfactometry data. J. Agric. Food Chem. 53(14): 5682-5690

Chafer A, Pascual-Martí MC, Salvador A, Berna A (2005). Supercritical fluid extraction and HPLC determination of relevant polyphenolic compounds in grape skin. J. Sep.Sci. 28(16): 2050-2056

Escudero A, Asensio E, Cacho J, Ferreira V (2002). Sensory and chemical changes of young white wines stored under oxygen. An assessment of the role played by aldehydes and some other important odorants. Food Chem. 77(3): 325-331

Escudero A, Campo E, Fariña L, Cacho J, Ferreira V (2007). Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. J. Agric. Food Chem. 55(11): 4501-4510

Etievant PX (1991). Wine. In: H. Maarse (eds). Volatile compounds in foods and beverages. Marcel Dekker, Inc., New York., pp 483-546

Fernández CM, Ramos MJ, Pérez A, Rodríguez JF (2010). Production of biodiesel from winery waste: Extraction, refining and transesterification of grape seed oil. Bioresource Technol. 101(18): 7030-7035

Fiori L (2007). Grape seed oil supercritical extraction kinetic and solubility data: Critical approach and modeling. J. Supercrit. Fluid 43(1): 43-54

Gomez E, Martinez A, Laencina J (1994). Localization of free and bound aromatic compounds among skin, juice and pulp fractions of some grape varieties. Vitis 33: 1/4

González-Centeno MR, Rosselló C, Simal S, Garau MC, López F, Femenia A (2010). Physico-chemical properties of cell wall materials obtained from ten grape varieties and their byproducts: Grape pomaces and stems. Food Sci. technol-LEB 43(10): 1580-1586

Guendez R, Kallithraka S, Makris DP, Kefalas P (2005). Determination of low molecular weight polyphenolic constituents in grape (Vitis vinifera sp.) seed extracts: Correlation with antiradical activity. Food Chem. 89(1): 1-9

Hashizume K, Samuta T (1997). Green Odorants of Grape Cluster Stem and Their Ability to Cause a Wine Stemmy Flavor. J. Agric. Food Chem. 45(4): 1333-1337

Hernandez-Orte P, Cersosimo M, Loscos N, Cacho J, Garcia-Moruno E, Ferreira V (2009). Aroma development from non-floral grape precursors by wine lactic acid bacteria. Food Res. Int. 42(7): 773-781

Hernández-Orte P, Cersosimo M, Loscos N, Cacho J, Garcia-Moruno E, Ferreira V (2008). The development of varietal aroma from non-floral grapes by yeasts of different genera. Food Chem. 107(3): 1064-1077

Hogan S, Canning C, Sun S, Sun X, Zhou K (2010). Effects of grape pomace antioxidant extract on oxidative stress and inflammation in diet induced obese mice. J. Agric. Food Chem. 58(21): 11250-11256

Igartuburu JM, Pando E, Rodríguez-Luis F, Gil-Serrano A (1998). Structure of a hemicellulose B fraction in dietary fiber from the seed of grape variety palomino (Vitis vinifera cv. Palomino). J. Nat. Prod. 61(7): 881-886

Ju ZY, Howard LR (2003). Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin. J. Agric. Food Chem. 51(18): 5207-5213

Loscos N, Hernández-Orte P, Cacho J, Ferreira V (2010). Evolution of the aroma composition of wines supplemented with grape flavour precursors from different varietals during accelerated wine ageing. Food Chem. 120(1): 205-216

Lou X, Janssen HG, Cramers CA (1997). Parameters Affecting the Accelerated Solvent Extraction of Polymeric Samples. Anal. Chem. 69(8): 1598-1603

Louli V, Ragoussis N, Magoulas K (2004). Recovery of phenolic antioxidants from wine industry by-products. Bioresource Technol. 92(2): 201-208

Luque-Rodríguez JM, Luque de Castro MD, Pérez-Juan P (2007). Dynamic superheated liquid extraction of anthocyanins and other phenolics from red grape skins of winemaking residues. Bioresource Technol. 98(14): 2705-2713

Maicas S, Mateo JJ (2005). Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: A review. Appl.Micrbiol..Biot. 67(3): 322-335

Maier T, Göppert A, Kammerer DR, Schieber A, Carle R (2008). Optimization of a process for enzyme-assisted pigment extraction from grape (Vitis vinifera L.) pomace. Eur. Food Res.Technol. 227(1): 267-275

Makris DP, Boskou G, Chiou A, Andrikopoulos NK (2008). An investigation on factors affecting recovery of antioxidant phenolics and anthocyanins from red grape (Vitis vinifera L.) pomace employing water/ethanol-based solutions. Am. J. Food Tec. 3(3): 164-173

Monrad JK, Howard LR, King JW, Srinivas K, Mauromoustakos A (2010). Subcritical solvent extraction of anthocyanins from dried red grape pomace. J. Agric. Food Chem. 58(5): 2862-2868

Palma M, Taylor LT, Zoecklein BW, Douglas LS (2000). Supercritical fluid extraction of grape glycosides. J. Agric. Food Chem. 48(3): 775-779

Park SK, Morrison JC, Adams DO, Noble AC (1991). Distribution of free and glycosidically bound monoterpenes in the skin and mesocarp of muscat of alexandria grapes during development. Journal of Agricultural and Food Chemistry® 39(3): 514-518

Pérez-Jiménez J, Sáyago-Ayerdi SG (2009). Grape antioxidant dietary fibre: A natural food additive and a dietary supplement. Agro Fod Ind.Hi Tec. 20(3 SUPPL.): 22-24

Pinelo M, Rubilar M, Jerez M, Sineiro J, Núñez MJ (2005). Effect of solvent, temperature, and solvent-to-solid ratio on the total phenolic content and antiradical activity of extracts from different components of grape pomace. J. Agric. Food Chem. 53(6): 2111-2117

Richter BE, Jones BA, Ezzell JL, Porter NL, Avdalovic N, Pohl C (1996). Accelerated solvent extraction: A technique for sample preparation. Anal. Chem. 68(6): 1033-1039

Ruberto G, Renda A, Amico V, Tringali C (2008). Volatile components of grape pomaces from different cultivars of Sicilian Vitis vinifera L. Bioresource Technol. 99(2): 260-268

Sánchez Palomo E, Pérez-Coello MS, Díaz-Maroto MC, González Viñas MA, Cabezudo MD (2006). Contribution of free and glycosidically-bound volatile compounds to the aroma of muscat "a petit grains" wines and effect of skin contact. Food Chem. 95(2): 279-289

Strauss CR, Wilson B, Williams PJ (1987). 3-oxo-α-ionol, vomifoliol and roseoside in Vitis vinifera fruit. Phytochemistry 26(7): 1995-1997

Ugliano M, Henschke PA (2009). Yeasts and wine flavour. In: M. V. Moreno-Arribas and M. C. Polo (eds). Wine chemistry and biochemistry. Springer, New York, pp 313-392

Vasserot Y, Arnaud A, Galzy P (1993). Evidence for Muscat marc monoterpenol glucosides hydrolysis by free or immobilized yeast β-glucosidase. Bioresource Technol. 43(3): 269-271

Wilson B, Strauss CR, Williams PJ (1984). Changes in free and glycosidically bound monoterpenes in developing Muscat grapes. J. Agric. Food Chem. 32(4): 919-924

Publicación 8. Capacidad de la microbiota oral humana para producir agliconas vínicas odorantes a partir de precursores glicosídicos de uva.

Ability of human oral microbiota to produce wine odorant aglycones from odorless grape glycosidic aroma precursors

Carolina Muñoz-González, Carolina Cueva, M. Ángeles Pozo-Bayón, M. Victoria Moreno-Arribas

Publicación enviada

Abstract

Grape aroma precursors are odorless glycosides that represent a natural reservoir of potential active odorant molecules in wines. Since the first step of wine consumption starts on the oral cavity, the processing of these compounds in the mouth could be an important factor that might influence aroma perception. Therefore, the objective of this work has been to evaluate the role of bacteria from the oral cavity on the transformation of odorless glycoside wine aroma precursors isolated from grapes in the corresponding odorant aglycones. To do so, in a first experiment, the ability of nine representative oral bacteria, including aerobe, facultative anaerobe and obligate anaerobe species to hydrolyse glycoside precursors was tested. Then, the same experimental procedure was carried out but using human oral microbiota isolated from the saliva of healthy volunteers. Odorant aglycones were isolated from the cultures mediums and analysed by HS-SPME-GC/MS. Results showed the ability of oral microorganisms to hydrolyse grape aroma precursors releasing the corresponding terpenes, benzenic compounds and lipid derivatives compounds. The hydrolytic activity seemed to be bacteria-dependent and it was subjected to large inter-individual variability. Overall, these findings suggest that human oral microbiota could play an important role on the perception of retronasal aroma during wine consumption.

Introduction

The aromatic profile of many wines depends on the varietal compounds of the grapes that have been employed in their production. These varietal compounds can be present in grapes as free volatile compounds and, in much higher concentrations, as aroma precursors (Baumes 2009). Among them, non-volatile sugar bound conjugates are odorless molecules which represent a natural reservoir of odorants compounds in wines which can be naturally and slowly released during wine aging, or intentionally released by using oenological enzymes during winemaking. The volatile compounds that could be released from glycosidic aroma precursors are mainly terpenes, C13 nor-isoprenoids, benzenic derivatives, volatile phenols and C6 compounds (Baumes 2009). These compounds are generally potent flavor compounds characterized by low odour thresholds and interesting sensory properties (Maicas and Mateo 2005). For example, in the case of terpenes, they could provide flowery notes that are characteristics of some grape varieties such as Muscat (Etievant 1991).

Although the composition of wine aroma (both, free and conjugate) and its impact on orthonasal aroma has been extensively studied (Guth 1997; Rapp 1998; Ferreira et al. 2000; Grosch 2001; Sarry and Gunata 2004; Escudero et al. 2007), the mechanisms involved on retronasal aroma released during wine consumption and its impact on aroma perception have received very little attention. Besides the wine matrix composition or the physico-chemical characteristics of the aroma compounds, other factors that can affect retronasal aroma are dependent on physiological parameters (oral microbiota, saliva composition, oral mucosa, temperature, in-mouth air cavity volumes changes, etc). The influence of some of these factors on retronasal aroma has been evaluated for other food matrices or in model food systems (Buettner 2004; Pionnier et al. 2004; Pionnier et al. 2004; Mishellany-Dutour et al. 2012; Muñoz-Gonzalez et al. 2014), and for example some *in vitro* studies with human saliva have demonstrated the role of salivary enzymes (β-glycosidases, esterases, etc.) on the degradation of free aroma volatiles (Buettner 2002; Buettner 2002; Lasekan 2013). Among these physiological factors, oral microbiota could be an important paremeter influencing retronasal aroma. In fact, it has been previously reported the ability of some oral anaerobic bacteria to hydrolise odorless cysteine-S-conjugates from onion, bell pepper and wine into their corresponding odorant thiols (Starkenmann et al. 2008), which

might be related with a delay in aroma perception, as it was already observed by Peynaud (Peynaud 1996) after the consumption of Golden Sauvignon grapes.

Oral microbiota is one of the most complex bacterial communities associated with the human body and it is formed by more than 700 bacterial species (Tian et al. 2010). These microorganisms can be present in the saliva or they can be adhered to oral surfaces and form an organized multispecies community known as biofilms (Kuramitsu et al. 2007). The main sources of nutrients for oral microbiota include saliva, crevicular fluid, and host diet. Although saliva is the main nutrient source due to its chemical composition and continuous production, the food is rich in a wide variety of components which could be used by the microbiota to generate secondary products. The relative short residence time of wine within the oral cavity, might invite to think in if so, a limited effect of oral microbiota on wine aroma perception. However, results from recent research (Munoz-Gonzalez et al. 2014), suggest a possible interaction of some wine matrix non volatile compounds with oral and pharyngeal mucosa which might increase the residence time of aroma precursors and free aroma compounds in the oral/pharyngeal cavities, thus, increasing their susceptibility to oral parameters (saliva, oral microbiota, etc). Moreover, the fact that during an *in vivo* consumption situation, wine is continuously replenished in the oral cavity makes viable the idea that oral microbiota might have a role on wine aroma perception.

To our knowledge, the transformation of wine odorless glycosidic aroma precursors into odorant aglycones by human microbiota is unknown. To check this hypothesis, a glycosidic precursor extract isolated from white grapes was incubated with representative oral bacteria species and with human oral microbiota isolated from the saliva of healthy volunteers, therefore considering the complexity of the whole microbiota in the mouth. Odorant aglycones were isolated from the cultures and analysed by HS-SPME-GC/MS and chemometric tools were applied in order to gain insight on the effect of different experimental factors (bacteria type, growing requirements, incubation time, saliva treatment, intra/inter-individual differences) on the ability of oral bacteria to hydrolyse wine glycosidic aroma precursors.

Material and methods

Reagents and solvents

Solvents (ethanol, dicloromethane, pentane, ethyl acetate and methanol) were obtained from Merck (Darmstadt, Germany) and LabScan (Gliwice, Poland). Pure water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Sodium chloride, sodium phosphate dibasic and sodium phosphate monobasic monohydrate came from Panreac (Barcelona, Spain) and Sigma-Aldrich (Steinheim, Germany).

Preparation of white grape precursor extract

Methodologies based on the protocol already published by Rodriguez-Bencomo et al., (2013)(Jose Rodriguez-Bencomo et al. 2013) were followed to obtain the white grape aroma precursor extract. Briefly, grapes were destemmed, crushed and filtered and glycosidic aroma precursors were isolated by retention on Amberlite XAD-2 resins from Supelco (Bellefonte, PA, USA). The eluated precursors were then evaporated to dryness, dissolved in milli Q water and stored at -20 ºC.

Experiment 1: In vitro oral microbiota experiments

Bacterial strains and growth conditions

The oral bacteria assayed in this study and the growth conditions are described in **Table 1**. These bacteria species can be naturally present in the oral cavity (*Streptococcus sanguinis*, *Streptococcus oralis*, *Actinomyces naeslundii*) and commonly encountered in the supragingival plaque (*Streptococcus mutans*, *Veillonella dispar*, *Fusobacterium. nucleatum*)(Bik et al. 2010). Some of them may be found in some circumstances in the mouth of healthy individuals (*Streptococcus aureus*, *Enterococcus faecalis*). The anaerobe facultative and anaerobe microorganisms were incubated under 5% CO_2 atmosphere and in anaerobic chamber (nitrogen 90%, carbon oxide 5%, hydrogen 5%), respectively. All strains were cryo-preserved at -80ºC in a sterilized mixture of culture medium and glycerol (50:50, v/v).

Table 1. Oral bacteria assayed in this study.

Microorganisms	Growth conditions
Aerobe	
Staphylococcus aureus ATCC 25923	TSB, 24 h, 37ºC
Enterococcus faecalis V583 (clinical isolate)	
Anaerobe facultative	
Streptococcus sanguinis DSMZ 20567	Modified TSB, 24h, 37ºC
Streptococcus oralis CECT 907	BHI, 24 h, 37ºC
Streptococcus mutans CECT 479	
Actinomyces naeslundii CECT 806	Modified BHI, 24-48 h, 37ºC
Anaerobe	
Granulicatella adiascens DSMZ 9848	
Veillonella dispar DSMZ 20735	WC, 24-48 h, 37ºC
Fusobacterium nucleatum DSMZ 15643	

TSB: Triptic Soy Broth (Difco); modified TSB (TSB containing 0.3% yeast extract); BHI: Brain Heart Infusion (Difco); modified BHI (BHI containing 1% casein, 0.5% glucose and 0.5% yeast extract); WC: Wilkins Chalgren Anaerobe broth (Difco).

Wine odorless glycosidic aroma precursor biotransformation by isolated oral bacteria

As a previous step for the evaluation of biotransformation of glycosidic aroma precursors by the oral bacteria, we assessed the antimicrobial effect of the grape extract on the growth of selected oral bacteria. For this purpose, a microdilution method described by Cueva et al. (2010)(Cueva et al. 2010) with some modifications was used. Briefly, in sterile 96-well microtiter plates, 100 µl of extract were diluted with broth and placed into the well containing 100 µl of bacterial suspension (1×10^6 cfu/mL). To adjust for the interference by grape pigments, a parallel series of mixtures with uninoculated broth was prepared (blank samples). Finally, a growth control with 100 µL of broth plus 100 µL of bacterial suspension was included. After incubation at 37 ºC, the bacterial growth was determined by reading the absorbance at 550 nm. The inhibition percentage of test microorganisms were calculated by comparing the control growth with those obtained from cultures with grape extract.

After making spectrophotometric measurements, the contents of the 10 wells (two milliliters) microtiter plates were placed in a 20 ml headspace vial to analyze the release of volatile compounds. All the experiments were performed in duplicate.

Experiment 2: Ex vivo oral microbiota experiments

Collection of saliva

Unstimulated saliva samples were freshly collected from three healthy panelists, age between 28-31 years. The volunteers were nonsmokers and were undergoing no antibiotics and other medical treatments three months before sampling. Panelists were asked not to consume any food or drink 2 h before donating saliva and to spit directly into the saliva collection tube. Saliva samples were centrifuged at 2600 g for 10 min at 4 ºC to remove excessive mucus and dead cells (crude saliva). To recover the representative oral microbiota, fresh and unstimulated saliva was diluted in SHI medium (pH = 7). This is an optimal medium for culturing oral bacteria, which has been shown to be able to sustain the growth of diverse microbial communities with similar microbial profiles to the original salivary microflora (Tian et al. 2010). Half of the crude saliva was incubated in the growth medium under aerobic or anaerobic conditions, separately, and the other half was centrifuged longer (27000 g, 30 min, 4 ºC), pasteurized at 60 ºC for 30 minutes and clarified by a final centrifugation (27000 g, 30 min, 4 ºC) (sterile saliva). One part of the sterile saliva was pre-heated to 80 ºC from 15 minutes to destroy enzyme activity and cooled to 37 ºC (non-enzymatic saliva) (Kamonpatana et al. 2012). Therefore, four types of saliva were employed for this study (aerobic, anaerobic, sterile and non-enzymatic saliva). Bacterial counts were performed after plating on SHI agar in aerobic and anaerobic conditions at 37 ºC for 24-48 h. No bacteria were found in sterile and non-enzymatic saliva samples after anaerobic or aerobic incubation for 72 h at 37 ºC. Saliva samples were pooled or separately treated depending on the experiment.

Wine odorless glycosidic aroma precursor biotransformation by human oral microbiota

Two mL of each saliva type obtained as described above were independently inoculated into 10 ml of the SHI medium (Tian et al. 2010). The cultures were incubated under both aerobic and anaerobic conditions (nitrogen 90%, carbon oxide 5%, hydrogen 5%) at 37 ºC for 24 h. Then, the grape precursor extract were diluted in the saliva solutions, and the hydrolysis by oral microbiota was measured by analyzing the

release of volatile compounds at different times (0 h, 2 h, 24 h, 72 h). All the experiments were performed in triplicate.

HS-SPME procedure

Two milliliters of sample were placed into a 20 ml headspace vial that was sealed with a PTFE/silicone septum (Supelco, Bellefonte, PA, USA). Forty microliters of an internal standard solution (3-octanol 10 mg/L) and 0.5 g of NaCl were added to each vial. The extraction was automatically performed by using a CombiPal system (CTC Analytics) provided with a 50/30 µm DVB/CAR/PDMS fiber of 2 cm length (Supelco, Bellefonte. PA). The samples were previously incubated for 5 minutes at 30 ºC and the extraction was performed in the headspace of the vial for 5 minutes at 30 ºC. The desorption was performed in the injector of the GC chromatograph (Agilent 6890N, Agilent, Palo Alto, CA) in splitless mode for 1.5 minutes at 270 ºC. After each injection the fiber was cleaning for 30 minutes avoiding any memory effect.

Gas Chromatography–Mass Spectrometry analysis

An Agilent MSD ChemStation Software was used to control the system. For separation, Supra-Wax fused silica capillary column (60 m × 0.25 mm i.d. × 0.50 µm film thickness) from Konik (Barcelona, Spain). Helium was the carrier gas at a flow rate of 1 ml/min. The oven temperature was programmed as follows: 40 ºC as initial temperature, held for 5 minutes, followed by a ramp of temperature at 4 ºC/min to 240 ºC and then held for 20 minutes.

For the MS system (Agilent 5973N), the temperatures of the transfer line, quadrupole and ion source were 270, 150 and 230 ºC respectively; electron impact mass spectra were recorded at 70 eV ionization voltages and the ionization current was 10 µA. The acquisitions were performed in Scan (from 35 to 350 amu) and SIM mode for some specific compounds. The identification of compounds was carried out by comparison of retention times and mass spectra of the references compounds with those reported in the mass spectrum library NIST 2.0.

Statistical analysis

Principal component analysis (PCA) was applied to examine the relationship between odorant aglycones release data and the isolated bacteria assayed. One-way

ANOVA and LSD test were carried out with free aroma released data to gain insight on the impact of aerobic and anaerobic cultures on the hydrolysis of glycosides aroma precursors. Aroma release data from the three panelists were separately treated and submitted to three-way ANOVA to determine significant effects of the studied factors: incubation time, saliva type and individuals (only considering the main effects). The significance level was $P < 0.05$ throughout the study. The STATISTICA program for Windows version 7.1 was used for data processing (StatSoft, Inc., 2005, www.statsoft.com).

Results and discussion

The ability of human oral microbiota to hydrolyse odorless glycosidic aroma precursors was studied by using two different approaches. In the first one, *in vitro* experiments with representative bacteria isolated from the oral cavity were performed (**experiment 1**), while the second approach consisted in *ex-vivo* experiments using the whole oral microbiota isolated from the saliva of three individuals (**experiment 2**). The main results are explained in the following sections.

Ability of some representative oral bacteria to release free volatiles from grape odorless glycosidic aroma precursors (Experiment 1)

To evaluate the role of oral bacteria on the release of free odorant volatiles, representative oral bacteria were selected and grown in their specific culture broth (**Table 1**) containing the aroma precursor extract isolated from grapes. Preliminary tests were performed in order to establish if the glycoside concentration employed in these experiments might inhibit the bacterial growth. From these results it was demonstrated that none of the oral bacteria assayed were inhibited by the glycosidic extract at the assayed concentration. Odorant aglycones were isolated from the cultures and analysed by HS-SPME-GC-MS. All aroma release data (aglycones produced from the precursor extract) were previously corrected taking into consideration the compounds generated in the control samples (aroma precursor extract incubated in the same broths but without the presence of bacteria). As shown in **Table 2** all the oral bacteria species assayed were able to hydrolyze grape glycosides releasing different types of aglycones belonging to different chemical families (terpenes, benzenic derivatives and C6-alcohols). On the basis of their aroma characteristics some of these compounds might be relevant for aroma perception. For instance, terpenes are important odorant compounds that exhibit

Table 2. Ability of different types of oral bacteria to produce aromatic aglycones from odorless grape glycosidic precursors. Data represent the comparison of the same aroma compound determined in different microbial cultures and are expressed in percentage considering the highest value of a specific compound as 100% and comparing this value with the amounts of the same compound determined in the other microbial cultures.

	E. faecalis	*S. aureus*	*S. mutans*	*S. oralis*	*S. sanguinis*	*A. naeslundii*	*G. adiascens*	*V. dispar*	*F. nucleatum*
TERPENES									
Limonene	**100.00**	62.54	16.75	10.40	23.08	85.03	28.01	n.d	n.d
Linalool	84.20	12.94	17.50	23.00	38.99	**100.00**	54.74	0.70	2.13
α-terpineol	**100.00**	64.04	26.95	9.19	22.25	84.78	22.43	n.d	0.66
Nerol	**100.00**	38.23	37.89	n.d	76.39	64.48	n.d	n.d	n.d
Geraniol	**100.00**	47.19	36.54	n.d	92.82	70.83	n.d	n.d	n.d
cis-linalool oxide	21.28	0.15	31.72	0.23	3.20	**100.00**	n.d	n.d	n.d
trans-linalool oxide	20.34	9.54	35.84	n.d	5.19	**100.00**	n.d	n.d	n.d
β-myrcene	**100.00**	49.72	16.20	6.02	24.06	54.30	25.58	n.d	n.d
β-trans-ocimene	**100.00**	46.82	12.45	n.d	12.61	56.16	27.28	n.d	n.d
β-cis-ocimene	**100.00**	40.86	12.70	n.d	n.d	55.06	36.25	n.d	n.d
BENZENIC DERIVATIVES									
Benzyl alcohol	47.72	31.23	98.00	1.12	11.66	**100.00**	n.d.	5.26	n.d
β-phenylethyl alcohol	56.32	15.04	**100.00**	34.60	56.40	58.06	n.d.	10.69	0.92
C6 ALCOHOLS									
1-hexanol	**100.00**	12.27	90.80	n.d	11.05	14.50	1.14	3.61	0.21
cis-3-hexen-1-ol	90.22	37.98	**100.00**	n.d	24.01	43.52	n.d	n.d	n.d

n.d.: not detected

very low odour threshold and flowery-citric aroma nuances in wines(Baumes 2009). Linalool is one of the most common odorant aglycones released from some floral grape varieties such as Muscat, Riesling and Gewürztraminer, and it was found in all the cultures assayed. In addition, two benzenoids compounds (benzyl alcohol and *β*-phenylethyl alcohol) were also identified. Among them, *β*-phenylethanol could be particularly interesting since it has been related to rose-like odour. Furthermore, some lipids derivatives, such as C6-alcohols (1-hexanol), were identified in both the aerobic and anaerobic cultures. Nonetheless, other typical wine aroma compounds from grape glycosidic aroma precursors such as C-13 norisoprenoides, vanillins or volatile phenols (Baumes 2009) were not identified in the cultures.

As can be seen in **Table 2**, the ability to hydrolyse and release the corresponding odorant aglycones was different depending on the type of bacteria assayed (bacteria-dependent). For example, *A. naeslundii* was the major producer of linalool and their corresponding oxides suggesting that this microorganism could be the responsible for the generation of interesting floral and flowery notes. In general, *E. faecalis*, *A. naeslundii* and *S. mutans* were the highest releasers while *G. adiascens*, *V. dispar* and *F. nucleatum* were the lowest producers. Interestingly, the three latter bacteria are the obligate anaerobic cultures tested in this experiment, so these results seem to suggest that besides of the type of oral bacteria, the production of free aroma compounds might depend on the bacteria growth requirements (anaerobic, aerobic or facultative anaerobes cultures).

To further investigate whether there was a trend in the aglycone generation pattern, a principal component analysis (PCA) with the data of free aroma compounds produced from the glycosides precursors by the nine bacteria was performed. **Figure 1** shows the oral bacteria assayed in the plane defined by the two firsts principal components. PC1 explained the 62 % of data variability and it was highly and negatively correlated to all the monoterpenols except linalool oxides: |0.90| limonene, |0.79| linalool, |0.94| *α*-terpineol, |0.85| nerol, |0.82| geraniol, |0.91| *β*-myrcene, |0.87| *β-trans*-ocimene and |0.85| *β-cis*-ocimene and with |0.77| *cis*-3-hexen-1-ol. As can be seen in **Figure 1**, *A. naeslundii* and most of the streptococci assayed showed negative values for PC1, therefore, they showed the highest ability to release the above mentioned volatile compounds. However, the strict anaerobic bacteria (*G. adiascens*, *V. dispar* and *F. nucleatum*), presented positive values for PC1 and in consequence, low values for the

volatile compounds associated with it. Nonetheless, it has been previously reported that *F. nucleatum* present a good ability to hydrolise cystein-S-conjugates releasing the corresponding volatile thiol (Starkenmann et al. 2008). Another possible explanation to the low production of aromatic aglycones exhibited by these anaerobic microorganisms could be related to the difficulty of some of them, such as *F. nucleatum*, to growth in the commercial culture media (Rowland et al. 1987).

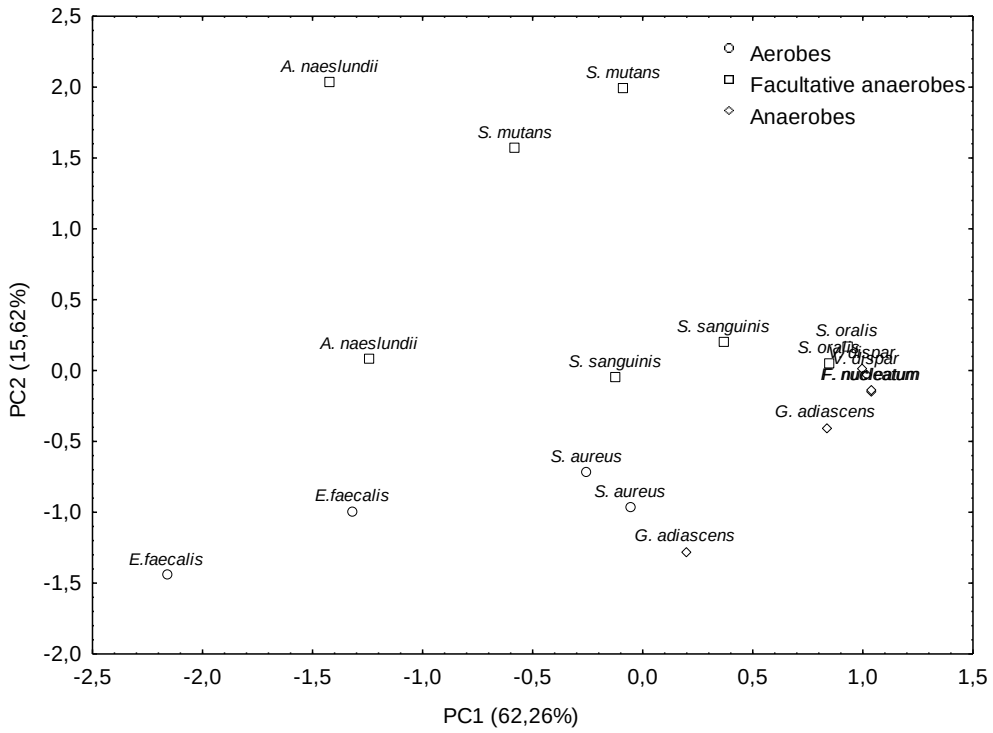


Figure 1. Principal component analysis (PCA) performed with the data corresponding to the odorant aglycones generated from grape glycosides by different oral bacteria.

To elucidate the significance of these results, it is necessary to understand the relevance of these bacteria in the human oral cavity. Oral cavity is a dynamic and heterogeneous system, which is composed of different environments. Surfaces in the oral cavity (as for example, teeth) are often colonized by bacterial biofilm (set of aerobic and anaerobic bacteria) that are frequently removed, by shedding of epithelial cells or by mechanical movement such as tooth brushing(Jakubovics and Kolenbrander 2010). However, oral bacterial are well adapted to recolonizing cleaning surfaces within minutes. The first bacteria to colonize the pellicle on the tooth surface are streptococci and actinomyces (Rickard et al. 2003; Li et al. 2004), and therefore, these microorganisms are predominant in the oral cavity during the early stages of the biofilm

formation (4-8 hours)(Li et al. 2004). This fact might have a special relevance because, as it has been observed in this work (**Figure 1),** these microorganisms were the highest producers of odorant aglycones from glycosidic precursors. Secondary colonizers, normally obligate anaerobic bacteria such as *F. nucleatum*, require the presence of the first colonizers to growth. Therefore, they usually appear in a more advanced stage in the biofilm development.

To our knowledge, this is the first study that has demonstrated the ability of several representative oral bacteria to hydrolyse grape glycoside aroma precursors. The next step in this study was to evaluate the ability of oral microorganisms to release odorant compounds from grape aroma precursors, but using an *ex vivo* approach considering the whole human oral microbiota, as it is explained in the following section.

Ability of human oral microbiota to release free volatiles from grape odorless glycosidic aroma precursors (Experiment 2)

To understand the role of human oral microbiota on the transformation of odorless glycosidic precursors into odorant molecules, fresh saliva samples from three different individuals (n = 3) were recovered, pooled and incubated overnight in both, aerobic and anaerobic conditions. The microbial counts obtained were 3.4 x 10^7 and 4.3 x 10^7 cfu/mL, in aerobic and anaerobic conditions, respectively. Then, the precursor extract was added and the kinetic of volatile generation (terpenes, benzenic derivatives and C6 compounds) was monitored by HS-SPME-GC/MS at different incubation times (0h, 2h, 24h and 72h). The same procedure was carried out but using the sterile (without microorganisms) and non-enzymatic (without microorganisms or enzymes) saliva samples. These saliva samples were used as controls for the bacterial hydrolysis potential. For each saliva sample three independent replicates were performed.

The aroma analysis showed that in agreement with results from **experiment 1**, oral salivary microorganisms incubated in both, aerobic and anaerobic conditions were able to hydrolyse grape glycosides releasing the corresponding aromatic aglycones (**Table 3**). Even more interesting was the fact that no odorant aglycones were determined in the samples incubated in presence of sterile or non-enzymatic saliva, suggesting that the glycosidic hydrolysis was only due to the action of oral microbiota.

Table 3. Average values and LSD test of odorant aglycones released from the grape precursors extract by oral human microbiota in aerobic and anaerobic conditions determined by HS-SPME-GC-MS at different incubation times.

	Aerobic saliva				Anaerobic saliva			
	0 h	**2 h**	**24 h**	**72 h**	**0 h**	**2 h**	**24 h**	**72 h**
	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
TERPENES								
Linalool	n.d a	60.85 a	1818.98 b	1764.47 b	n.d a	62.75 a	2298.79 b	2209.19 b
α-Terpineol	4.04 a	11.88 a	105.71 b	115.70 b	4.02 a	10.09 a	131.57 b	229.64 c
Nerol (*cis*-geraniol)	n.d a	n.d a	108.77 b	134.51 c	n.d a	0.00 a	185.51 b	236.39 c
Geraniol	n.d a	n.d a	131.63 c	78.65 b	n.d a	0.00 a	171.57 b	174.54 b
Limonene	n.d a	n.d a	86.90 b	83.30 b	n.d a	0.00 a	127.41 b	123.25 b
cis-linalool oxide	n.d a	n.d a	456.64 c	406.49 b	n.d a	29.07 a	473.74 b	648.35 c
trans-linalool oxide	n.d a	n.d a	54.11 c	46.61 b	n.d a	2.29 a	57.35 b	113.27 c
β-myrcene	n.d a	n.d a	343.61 b	326.95 b	n.d a	n.d a	488.03 b	466.36 b
β-*trans*-ocimene	n.d a	n.d a	45.80 b	41.47 b	n.d a	n.d a	66.95 b	62.19 b
β-*cis*-ocimene	n.d a	n.d a	78.67 b	79.76 b	n.d a	0.35 a	112.89 b	108.09 b
Sum	***4.04***	***95.20***	***3230.82***	***3077.90***	***4.02***	***104.54***	***4121.85***	***4371.27***
BENZENIC DERIVATIVES								
Benzyl alcohol	2.95 a	3.20 a	24.97 b	22.93 b	2.84 a	2.99 a	32.50 b	44.66 c
β-phenylethyl alcohol	8.74 a	8.74 a	60.39 b	185.71 c	6.94 a	6.46 a	37.25 b	62.68 c
Sum	***11.69***	***11.94***	***85.35***	***208.64***	***9.78***	***9.44***	***69.75***	***107.35***
C6 ALCOHOLS								
1-Hexanol	10.39 a	18.06 ab	94.08 c	23.17 b	13.33 a	20.70 a	124.26 b	147.02 c
cis-3-Hexen-1-ol	n.d a	n.d a	16.78 c	5.19 b	n.d a	n.d a	20.30 b	23.53 c
Sum	***10.39***	***18.06***	***110.86***	***28.36***	***13.33***	***20.70***	***144.56***	***170.55***

All values (area: arbitrary unit) are divided by a factor of 1.000. Different letters for the same aroma compound in the same growth conditions (aerobic, anaerobic) denote statistical differences among incubation times after applying LSD test. **n.d.: not detected**

A one-way ANOVA and a LSD test were also carried out with data from aerobic and anaerobic saliva samples at different incubation times (**Table 3).** This treatment allowed us to compare the effect of incubation time in both conditions. Interestingly, in spite of the well-known aroma retention effect produced by some saliva proteins(Starkenmann et al. 2008; Muñoz-Gonzalez et al. 2014), we observed a significant increase of aromatic aglycones released from the odorless glycosides with an increase in the incubation time. Although after two hours of incubation there was an initial release of aromatic aglycones, the highest release was produced at longer incubation times (24 h or 72 h). The majority of aromatic aglycones released by aerobic cultures such as, some terpenes, all the C6-alcohols and benzyl alcohol reached the maximum concentration values after 24 h of incubation (**Table 3**). The rest of compounds were more abundant after 72 h of incubation time. In contrast, the anaerobic cultures seemed to act slowly (as previously shown in **experiment 1**) and the highest values for most odorants were observed after 72 h of incubation. These differences could be due to the changes in the microbial population in the saliva samples during the experiment. It is probably that the bacterial composition in the fresh saliva samples would be formed by early microbial colonizers such as streptococci and actinomyces. In particular, it is known the predominance of the anaerobe facultative *A. naeslundii* strains in fresh saliva samples(Li et al. 2004), which could be present in both aerobic and anaerobic saliva samples. These initial colonizers might provide adequate environmental conditions for subsequent colonizers, and therefore an increase in the growth of the obligate anaerobic, which could explain the higher release of volatile aglycones in these samples versus aerobic samples. Although 24 h or 72 h could be considered as long incubation times to observe the effect produced by oral microbiota, it is also important to keep in mind that oral mucosa might retain food non volatile compounds(Buettner et al. 2001), (such as glycosides) increasing their residence time in the oral cavity, therefore making them more susceptible to oral physiological factors, such as oral microbiota. Moreover, many of the odorant aglycones released from glycosidic precursors present very low odour thresholds, therefore, very small concentrations of these volatiles might have a large impact on aroma perception, However, further experiments are needed in order to validate this hypothesis.

Table 3 also shows that even if either aerobic or anaerobic microbiota were able to hydrolyse glycoside precursors, the extent of this effect was different from each of

them. For instance, anaerobic microbiota from saliva produced the highest levels of terpenes and C6-alcohols, however benzenic compounds were higher released in the aerobic cultures. Interestingly, in **Table 3** it can also be observed a significant decrease of C6-alcohols after 24h of incubation in aerobic saliva, which suggest a possible metabolisation of these compounds by aerobic bacteria, which also will deserve more attention in future research.

On the other hand, the human mouth is home to a large number of microbial communities and recently, it has been highlighted that there are significant interindividual differences at the species and strain level in the composition of human oral microbiota(Bik et al. 2010). Therefore, a second experiment was carried out in order to investigate interindividual differences on the ability of representative human oral microbiota to release free volatiles from grape odorless glycosidic aroma precursors. To do so, the same procedure previously described was conducted with the microbiota isolated from the saliva of three individuals that was separately treated. Experiments were performed in triplicate. Data corresponding to absolute peak areas of the aroma compounds determined in these samples were submitted to a three-way ANOVA (only considering the main effects): incubation time (0h, 2h, 24h, 72h), interindividual variability (n=3) and type of saliva (sterile, non-enzymatic, aerobic and anaerobic saliva). From the three studied factors, all the compounds (aromatic aglycones) presented significant differences (data not shown).

In addition, results corresponding to the evolution of the amount of aroma produced (absolute peak area) over time were plotted to obtain the aroma release profile. An example corresponding to the release of linalool at the essayed times is shown in **Figure 2**. As it can be seen, the three individuals exhibited significant differences on the formation of linalool over incubation time. In the case of the aerobic culture, panelist #1 exhibited the highest aroma formation, while individuals #2 and #3 did not show a great difference between them. For the anaerobic culture, individual #1 showed the highest aroma formation but individual #3 showed a great increase after 72 hours of incubation time. This seems to be related to the above results, and indicate than the obligate anaerobe microorganisms present in the anaerobic saliva sample could require more time to growth. In order to discard that the observed differences in the release patterns of linalool were due to the quantitative differences in the number of

microorganisms initially present in the saliva samples from among individuals, a seeding of the original microbiota of the saliva samples and subsequent counting in SHI agar plates was carried out. The counts obtained were very similar for all 3 individuals, nearly to 1 *x* 10^7 cfu/ml, suggesting that the observed differences were effectively due to the specific bacterial composition of the saliva from each individual (qualitative differences). This result is in agreement with that observed in **experiment 1**, indicating once again that the type of bacteria is a very important factor to explain the ability to hydrolyse glycosidic aroma precursors and the formation of the corresponding odorant compound. These large interindividual differences observed are in agreement with previous results(Walle et al. 2005), indicating that oral hydrolysis (and therefore volatile release) may be different for each individual, which could be an important aspect when trying to understand differences in aroma perception.

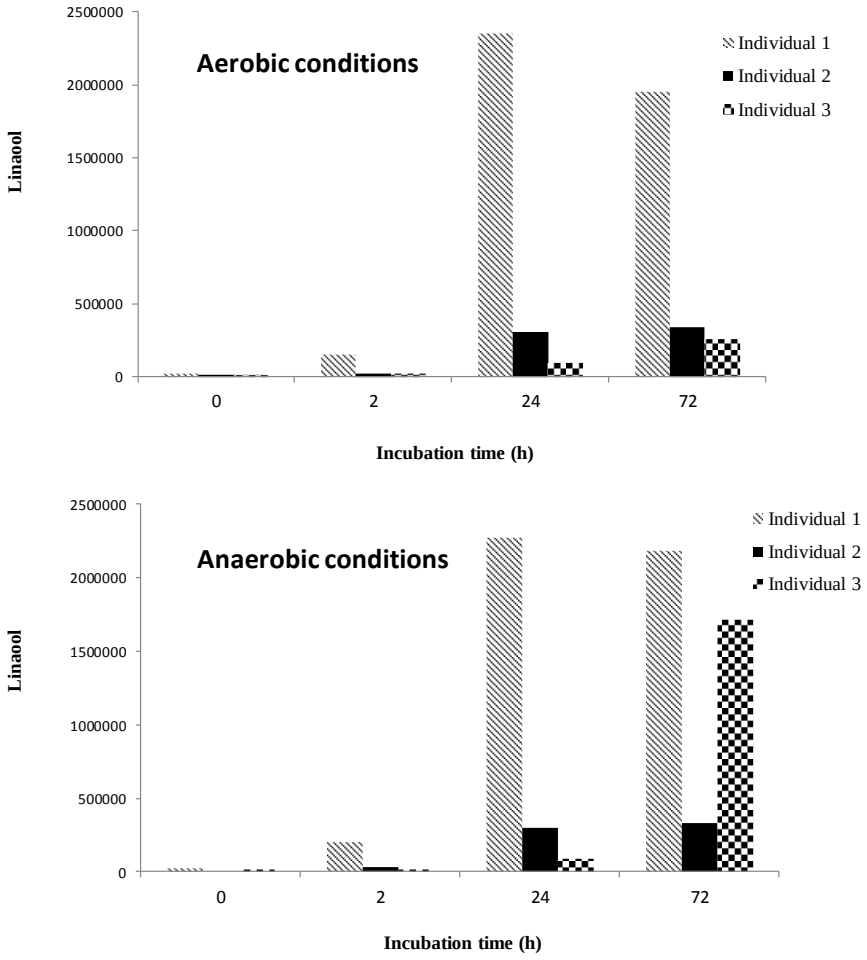


Figure 2. Linalool produced (absolute peak areas) from grape glycosidic aroma precursors by aerobic and anaerobic cultures isolated from human saliva at different incubation times.

In summary, in this study the ability of oral microbiota to release varietal aroma compounds from odorless grape glycosides has been proven. This capacity is bacteria-dependent and related to their growth requirements. Another important finding of this work was the fact that odorant aglycones were only produced during the incubation of the aroma precursor extract with oral microbiota isolated from fresh saliva samples, but odorant release was not produced when the incubation was performed with saliva enzymes. In addition, a high inter-individual variation in the oral microbiota hydrolytic performance was observed, which seem to be more linked to qualitative differences in microbiota composition than to quantitative differences. Finally, it is important to consider that the extent of the hydrolytic effect of human oral microbiota could be higher in *in vivo* conditions, than in the experimental *in vitro* conditions used in this study. More experiments are necessary in order to determine the meaning of this effect on retronasal aroma perception during wine consumption.

References

Baumes R (2009). Wine aroma precursors. In: P. M. Moreno-Arribas MV (eds). Wine chemistry and biochemistry. Springer, New York

Bik EM, Long CD, Armitage GC, Loomer P, Emerson J, Mongodin EF, Nelson KE, Gill SR, Fraser-Liggett CM, Relman DA (2010). Bacterial diversity in the oral cavity of 10 healthy individuals. Isme Journal 4(8): 962-974

Buettner A (2002). Influence of human saliva on odorant concentrations. 2. aldehydes, alcohols, 3-alkyl-2-methoxypyrazines, methoxyphenols, and 3-hydroxy-4,5-dimethyl-2(5H)-furanone. Journal of Agricultural and Food Chemistry 50(24): 7105-7110

Buettner A (2002). Influence of human salivary enzymes on odorant concentration changes occurring in vivo. 1. Esters and thiols. Journal of Agricultural and Food Chemistry 50(11): 3283-3289

Buettner A (2004). Investigation of potent odorants and afterodor development in two Chardonnay wines using the buccal odor screening system (BOSS). Journal of Agricultural and Food Chemistry 52(8): 2339-2346

Buettner A, Beer A, Hannig C, Settles M (2001). Observation of the swallowing process by application of videofluoroscopy and real-time magnetic resonance imaging-consequences for retronasal aroma stimulation. Chemical Senses 26(9): 1211-1219

Cueva C, Victoria Moreno-Arribas M, Martin-Alvarez PJ, Bills G, Francisca Vicente M, Basilio A, Lopez Rivas C, Requena T, Rodriguez JM, Bartolome B (2010). Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. Research in Microbiology 161(5): 372-382

Escudero A, Campo E, Farina L, Cacho J, Ferreira V (2007). Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. Journal of Agricultural and Food Chemistry 55(11): 4501-4510

Etievant P (1991). Wine. In: M. H (eds). Volatile compounds in foods and beverages. Marcel Dekker, Inc, New York,

Ferreira V, Lopez R, Cacho JF (2000). Quantitative determination of the odorants of young red wines from different grape varieties. Journal of the Science of Food and Agriculture 80(11): 1659-1667

Grosch W (2001). Evaluation of the key odorants of foods by dilution experiments, aroma models and omission. Chemical Senses 26(5): 533-545

Guth H (1997). Quantitation and sensory studies of character impact odorants of different white wine varieties. Journal of Agricultural and Food Chemistry 45(8): 3027-3032

Jakubovics NS, Kolenbrander PE (2010). The road to ruin: the formation of disease-associated oral biofilms. Oral Diseases 16(8): 729-739

Jose Rodriguez-Bencomo J, Selli S, Munoz-Gonzalez C, Martin-Alvarez PJ, Angeles Pozo-Bayon M (2013). Application of glycosidic aroma precursors to enhance the aroma and sensory profile of dealcoholised wines. Food Research International 51(2): 450-457

Kamonpatana K, Giusti MM, Chitchumroonchokchai C, MorenoCruz M, Riedl KM, Kumar P, Failla ML (2012). Susceptibility of anthocyanins to ex vivo degradation in human saliva. Food Chemistry 135(2): 738-747

Kuramitsu HK, He X, Lux R, Anderson MH, Shi W (2007). Interspecies interactions within oral microbial communities. Microbiology and Molecular Biology Reviews 71(4): 653-+

Lasekan O (2013). A Comparative Analysis of the Influence of Human Salivary Enzymes on Odorant Concentration in Three Palm Wines. Molecules 18(10): 11809-11823

Li J, Helmerhorst EJ, Leone CW, Troxler RF, Yaskell T, Haffajee AD, Socransky SS, Oppenheim FG (2004). Identification of early microbial colonizers in human dental biofilm. Journal of Applied Microbiology 97(6): 1311-1318

Maicas S, Mateo JJ (2005). Hydrolysis of terpenyl glycosides in grape juice and other fruit juices: a review. Applied Microbiology and Biotechnology 67(3): 322-335

Mishellany-Dutour A, Woda A, Laboure H, Bourdiol P, Lachaze P, Guichard E, Feron G (2012). Retro-Nasal Aroma Release Is Correlated with Variations in the In-Mouth Air Cavity Volume after Empty Deglutition. Plos One 7(7)

Munoz-Gonzalez C, Martin-Alvarez PJ, Victoria Moreno-Arribas M, Angeles Pozo-Bayon M (2014). Impact of the Nonvolatile Wine Matrix Composition on the In Vivo Aroma Release from Wines. Journal of Agricultural and Food Chemistry 62(1): 66-73

Muñoz-Gonzalez C, Feron G, Guichard E, Rodriguez-Bencomo J, Alvarez PM, Moreno-Arribas M, Pozo-Bayon M (2014). Understanding the role of saliva on aroma release from wine by using static and dynamic headspace conditions Submitted

Peynaud E (1996). In: (eds). The taste of wine: tha art and science of wine appreciation Wiley, New York, pp 66

Pionnier E, Chabanet C, Mioche L, Le Quere JL, Salles C (2004). 1. In vivo aroma release during eating of a model cheese: Relationships with oral parameters. Journal of Agricultural and Food Chemistry 52(3): 557-564

Pionnier E, Chabanet C, Mioche L, Taylor AJ, Le Quere JL, Salles C (2004). 2. In vivo nonvolatile release during eating of a model cheese: Relationships with oral parameters. Journal of Agricultural and Food Chemistry 52(3): 565-571

Rapp A (1998). Volatile flavour of wine: Correlation between instrumental analysis and sensory perception. Nahrung-Food 42(6): 351-363

Rickard AH, Gilbert P, High NJ, Kolenbrander PE, Handley PS (2003). Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends in Microbiology 11(2): 94-100

Rowland MD, Delbene VE, Lewis JW (1987). Factors affecting antimicrobial susceptibility of fusobacterium species. journal of clinical microbiology 25(3): 476-479

Sarry JE, Gunata Z (2004). Plant and microbial glycoside hydrolases: Volatile release from glycosidic aroma precursors. Food Chemistry 87(4): 509-521

Starkenmann C, Le Calve B, Niclass Y, Cayeux I, Beccucci S, Troccaz M (2008). Olfactory Perception of Cysteine-S-Conjugates from Fruits and Vegetables. Journal of Agricultural and Food Chemistry 56(20): 9575-9580

Tian Y, He X, Torralba M, Yooseph S, Nelson KE, Lux R, McLean JS, Yu G, Shi W (2010). Using DGGE profiling to develop a novel culture medium suitable for oral microbial communities. Molecular Oral Microbiology 25(5): 357-367

Walle T, Browning AM, Steed LL, Reed SG, Walle UK (2005). Flavonoid glucosides are hydrolyzed and thus activated in the oral cavity in humans. Journal of Nutrition 135(1): 48-52

Discusión general

5. DISCUSIÓN GENERAL

La producción de vinos competitivos en el mercado nacional e internacional y la posibilidad de adaptarlos a los requerimientos de los consumidores actuales requiere de estudios científicos encaminados a incrementar la calidad de los mismos. En este sentido, una de las características clave que determina la calidad de un vino es su aroma. No es de extrañar por tanto que, como se ha comentado en distintos momentos a lo largo de esta Memoria, la mayor parte de los esfuerzos encaminados a explicar el aroma de los vinos se haya centrado en la caracterización del perfil volátil y en intentar elucidar el impacto odorante de cada uno de estos constituyentes en los vinos (Rapp y Mandery, 1986; Campo y col, 2006a; Campo y col., 2006b; Campo y col., 2007; Culleré y col., 2008; Polaskova y col., 2008; Ferreira y col., 2009; Ebeler y col., 2009; Styger y col., 2011, Muñoz-González y col., 2011, Villamor y col., 2013). Pese a todo, la relación entre la composición global de un vino y la percepción del aroma durante el consumo sigue siendo difícil de explicar (Styger et al., 2011). Esto se puede deber a diferentes motivos: a) a la presencia de compuestos odorantes en el vino que aún no se han identificado con las actuales técnicas analíticas (Ferrira y Cacho, 2009), b) a que se desconoce el impacto de la fisiología humana en la liberación del aroma durante el consumo o c) o a que en muchos de los estudios no se ha tenido en cuenta el papel que puede ejercer la composición no volátil del vino (matriz vínica) en la liberación del aroma durante el consumo (Muñoz-González y col., 2011)

Enmarcado en este contexto, el presente trabajo de Tesis Doctoral ha tenido como objetivo estudiar cómo se produce la liberación del aroma durante el consumo, teniendo en cuenta el efecto de la composición no volátil del vino (matriz del vino) y de factores relacionados con la fisiología oral (saliva, microbiota oral, mucosa oral). Estos parámetros pueden influir en la composición del aroma retronasal y, en última instancia, ejercer un impacto en la percepción del aroma del vino.

En una primera etapa del trabajo, se decidió comprobar si la composición de la matriz vínica podría influir en la liberación del aroma del vino. Para ello, se determinó la capacidad de retención de matrices no volátiles procedentes de vinos de diferente tecnología de elaboración sobre un amplio grupo de compuestos (36 compuestos) representativos y característicos de las distintas familias que integran el aroma del vino. Uno de los aspectos más originales del estudio con respecto a otros previamente

publicados en la literatura, es haber considerado el efecto de la composición global de la matriz vínica en lugar de emplear soluciones vínicas sintéticas evaluando un número limitado de macromoléculas y de compuestos del aroma (Voilley y col., 1991; Jung y Ebeler 2003; Aronson y Ebeler, 2004; Whiton y Zoecklein, 2000; Hartmann y col., 2002; Chalier y col., 2007). En este estudio (**Publicación 1**), se empleó la técnica de SPME para monitorizar el espacio de cabeza en condiciones estáticas, sobre cinco matrices vínicas (blanco, espumoso, dulce, tinto joven y tinto crianza) reconstituidas a la misma concentración de etanol y aromatizadas con compuestos de aroma representativos del perfil volátil del vino. Con este procedimiento se aseguró que el etanol no afectara de forma diferente la volatilidad de los compuestos volátiles al espacio de cabeza, y por lo tanto, las diferencias observadas entre vinos fueron exclusivamente debidas a diferencias en su composición.

Este estudio puso de manifiesto por primera vez, el impacto de la matriz vínica en la volatilidad de la mayoría de los compuestos del aroma mediante dos efectos bien diferenciados. El principal efecto observado fue la retención de los compuestos volátiles por los componentes de la matriz vínica. Este efecto fue más importante en el vino espumoso, lo que pudo ser debido a la presencia de manoproteínas de levaduras que son abundantes en este tipo de vinos (Núñez y col., 2005), y que como se ha demostrado previamente, son capaces de retener varios tipos de compuestos de aroma (Chalier y col., 2007). Por otro lado, se observó un aumento en la volatilidad (efecto *salting-out*) de algunos de los compuestos ensayados, principalmente en los vinos de composición más compleja (dulce y tinto crianza). Este efecto pudo ser debido a la presencia de algunos componentes (como los mono- y disacáridos) que podrían captar moléculas de agua, disminuyendo la cantidad de agua libre disponible, lo que podría afectar el equilibrio de los volátiles aumentando su presencia en el espacio de cabeza (Delarue y Giampaoli, 2006). Además, los efectos observados fueron dependientes de las propiedades físico-químicas (volatilidad e hidrofobicidad) de cada compuesto de aroma. Por ejemplo, los ésteres y terpenos mostraron un fuerte efecto de retención en la matriz. Sin embargo, los alcoholes prácticamente no se vieron afectados. Además, es de remarcar que dentro de un mismo grupo químico (como los C13-norisoprenoides) se observaron diferentes efectos, mostrando la alta especificidad de algunas interacciones entre compuestos volátiles y compuestos no volátiles de la matriz vínica.

En consecuencia, los resultados de este estudio pusieron de manifiesto que las interacciones entre compuestos del aroma y la matriz vínica afectan la liberación de aroma, lo que podría tener consecuencias tanto en la percepción ortonasal (cuando olemos un vino), como retronasal (durante el consumo del mismo), y fue el punto de partida de la presente Tesis Doctoral.

Sin embargo, este estudio se llevó a cabo en condiciones estáticas, que aunque se han reconocido como las más idóneas para el estudio de interacciones (Friel y Taylor, 2001; Fabre y col., 2002) no representan la situación dinámica que se produce durante el consumo. Además, este tipo de aproximación no representa la situación que se produce durante el consumo ya que no permite tomar en consideración todos los procesos fisiológicos envueltos en este proceso (como por ejemplo, la deglución, flujos de aire, presencia de saliva, etc.). Por tanto, se decidió comprobar si el efecto de la matriz del vino también podría afectar la liberación del aroma en condiciones reales de consumo. Pese a que esta investigación está cobrando gran importancia para algunos alimentos (por ejemplo, los derivados lácteos), en el caso del vino, este es un área apenas explorada.

Para este trabajo se desarrolló un sistema de atrapamiento del aroma retronasal (RATD) (**Publicación 2**) que considera la influencia de la fisiología humana en el proceso de consumo, y especialmente el impacto de la deglución que es el principal mecanismo de liberación de moléculas del aroma durante el consumo de alimentos líquidos (Buettner y Schierberle, 2000; Buettner y col., 2001; Buettner y col., 2002a). Tras la deglución, este dispositivo permite atrapar en una trampa polimérica los compuestos volátiles liberados a nivel de las fosas nasales, que son una buena representación del aroma retronasal que interacciona con el sistema olfativo (Buettner y Schierberle, 2000). Posteriormente, los volátiles eran desorbidos y analizados por CIS-GC-MS. Las principales ventajas del empleo de un sistema de este tipo son la identificación precisa de los analitos de interés, que a veces es compleja en el caso del empleo de otras técnicas que permiten monitorizar la liberación de aroma *in vivo* (como la APCI-MS y PTR-MS), y la concentración de los que están muy diluidos en el aire exhalado, incrementando por tanto, la sensibilidad de la técnica (Buettner y Schierberle, 2000).

La aplicación de este sistema se realizó en vinos modelo de diferente composición (etanol y azúcar) lo que permitió comprobar el impacto de estos dos componentes, habituales en vinos y bebidas derivadas de vino, en la liberación del aroma en condiciones *in vivo* (**Publicación 2**). De esta manera, se comprobó que la adición de azúcar (150 mg/kg) no tuvo efecto sobre la liberación de aroma, mientras que la presencia de etanol, incluso a concentraciones relativamente bajas (40 mg/L) sí mostró un importante efecto, aumentando la liberación de aroma retronasal atrapado durante el consumo. Aunque el moderado efecto de azúcares simples como la sacarosa, empleada en la formulación de estos sistemas coincidía con trabajos previos en la literatura realizados en bebidas de lima-limón (Well y col., 2003), el resultado del efecto del etanol resultaba sorprendente y era el opuesto reportado en la mayoría de trabajos publicados con anterioridad (Voilley y col., 1991; Conner 1998; Whiton y Zoecklein, 2000, Hartmann y col., 2002; Camara y col., 2006). Sin embargo, la mayor parte de estos estudios se habían realizado en condiciones estáticas, en las que presencia de etanol aumenta la solubilidad de los compuestos del aroma disminuyendo por tanto su presencia en el espacio de cabeza (Aznar y col., 2004; Aprea y col., 2007). Por lo tanto, se extrajeron diferentes conclusiones a la hora de valorar el efecto de un ingrediente o componente de un alimento en la liberación del aroma dependiendo de las condiciones experimentales ensayadas (estáticas *vs* dinámicas). Para intentar explicar la mayor liberación de aroma en los vinos modelo con un mayor contenido de etanol se han propuesto diferentes hipótesis (Clark y col., 2011). La primera de ellas es que el etanol puede inducir cambios en la tensión superficial que afecta la distribución del líquido en la boca y faringe durante el consumo, lo que permite la formación de una superficie de contacto mayor, y por tanto una, mayor liberación de aroma. Otro efecto del etanol podría estar relacionado con su capacidad para aumentar la solubilidad de los compuestos del aroma en la mucosa (oral y faríngea) previniendo las pérdidas y/o aumentando la cantidad de compuestos volátiles en la interfase gas – líquido, lo que podría promover la liberación del aroma. Finalmente, el llamado efecto Marangoni (Hosoi y Bush, 2001), también podrían estar involucrado. En este caso, la evaporación del etanol en la interfase gas – líquido (en la boca y la garganta) podría crear una transferencia de nuevas moléculas de etanol y compuestos volátiles para reponer las pérdidas que se producen en la superficie, lo que podría aumentar la cantidad de aroma liberado (Tsachaki y col., 2005).

El trabajo anterior (**Publicación 2**) permitió, como se ha comentado, optimizar una metodología para la monitorización del aroma en condiciones *in vivo*, que resultaba muy interesante para evaluar el efecto de la matriz vínica en la liberación de aroma que, como se había comprobado es un factor importante en la liberación de aroma en condiciones estáticas (HS-SPME-GC/MS). Por ello, durante el siguiente estudio, se evaluó el efecto de la matriz vínica en la liberación de aroma in vivo empleando el previamente optimizado sistema de atrapamiento de aroma retronasal (**Publicación 3**).

Para ello, se emplearon de nuevo los cinco tipos de vino con distinta tecnología de elaboración (blanco, espumoso, tinto joven, tinto crianza y dulce) y por tanto composición, que fueron evaluados por seis voluntarios, a los que previamente se les entrenó en un procedimiento específico de consumo y familiarización con el RATD para tratar de minimizar las diferencias interindividuales. De nuevo, y como en estudios previos, para evitar el efecto del etanol en la volatilidad de los compuestos de aroma, todos los vinos (excepto el dulce) se ajustaron al mismo contenido de etanol y se aromatizaron con cuatro compuestos target volátiles de grado alimentario que estaban presentes en una adecuada concentración en los exhalados de respiración tras el consumo del vino.

Los resultados de este trabajo mostraron, en primer lugar, importantes diferencias interindividuales en la liberación del aroma durante el consumo que permitieron la agrupación de los panelistas en altos y bajos liberadores de aroma, lo que parecía estar directamente relacionado con diferencias en su capacidad respiratoria. Este resultado coincidía con el de otros autores obtenidos durante el consumo de quesos modelo (Pionnier y col., 2004) o emulsiones lipídicas (Frank y col., 2011). Principalmente en el grupo de los bajos liberadores de aroma, se observó una influencia significativa de la matriz del vino. En este grupo de panelistas, el consumo de vinos tintos producía una cantidad significativamente mayor de aroma liberado que el consumo de los vinos blancos y el dulce del estudio, a pesar de estar aromatizados al mismo nivel y presentar similares niveles de alcohol.

El hecho de que los vinos tintos liberaran mayor cantidad de aroma estaría relacionado con diferencias en su composición no volátil. La caracterización química de las matrices del estudio (pH, acidez total, polifenoles totales, polisacáridos neutros, azúcar residual y compuestos nitrogenados) y un estudio de correlación, permitió

comprobar que la variable más relacionada con el efecto observado fue la cantidad de polifenoles totales. Es conocido que los polifenoles, más abundantes y estructuralmente diferentes en vinos tintos que en vinos blancos, pueden interaccionar con los compuestos de aroma (Dufour y Bayonove, 1999a; Jung y col., 2000; Aronson y Ebeler, 2004; Villamor y col., 2013). El aumento de aroma liberado en vinos tintos y la relación con la cantidad de polifenoles totales del vino podría explicarse por la interacción de este tipo de compuestos no volátiles, más abundantes en vinos tintos, con las mucosas orales y faríngeas incrementando el área de contacto entre el aire y el vino tras el consumo, favoreciendo la liberación de aroma (Buettner y col., 2001). Por otra parte, en un reciente estudio, Mitropoulou y colaboradores (2011) han sugerido recientemente la formación de macrocomplejos formados por proteínas de la saliva, polifenoles y polisacáridos del vino capaces de encapsular moléculas de aroma hidrofóbicas en su interior. La formación de reservorios de aroma gracias a la formación de estos macrocomplejos, podrían retardar la liberación y posterior percepción del aroma tras el consumo. Este último mecanismo se ha propuesto para explicar la persistencia del aroma tras el consumo (Buettner 2004; Deleris y col., 2011).

Este estudio ha sido el primero en la literatura que ha demostrado el efecto de la matriz no volátil del vino en la liberación del aroma en condiciones *in vivo*. Aunque el comportamiento observado para los compuestos del aroma empleados en este estudio podría extrapolarse a otros compuestos integrantes del aroma del vino, sería interesante comprobar si este mismo efecto se puede observar en vinos sin aromatizar, es decir, empleando la composición aromática endógena del propio vino y así comprobar la relación de este efecto con la percepción sensorial. Además, sería interesante evaluar la naturaleza química de este tipo de interacciones que pueden ser diferentes en función del tipo de polifenol (monomérico, polimérico).

Los estudios anteriormente descritos, realizados tanto en condiciones estáticas (**Publicación 1**) como dinámicas (**Publicación 3**), simulando el consumo de vino proporcionaron importantes conclusiones acerca del impacto de la matriz vínica en la liberación del aroma. Sin embargo, durante la ingestión de un alimento, el aroma se libera de la matriz alimentaria dentro de la cavidad oral de manera secuencial. Esto hace que durante el tiempo que dura la ingestión de un alimento, podamos percibir diferentes sensaciones. Para considerar la dimensión temporal de la liberación del aroma que se produce durante el consumo, es necesario aplicar técnicas espectrométricas tales como

la APCI-MS o PTR-MS que permiten evaluar el aroma liberado en tiempo real y que pueden proporcionar una mejor correlación entre la sensación percibida durante el consumo y la concentración y el tipo de molécula responsable (Lindinger y col., 1998; Yereztian y col., 2000). Aunque estas técnicas se han utilizado para el estudio de liberación de aroma de distintos tipos de alimentos (Pionnier y col., 2004a; Feron y col., 2014), en el caso del vino se han empleado casi exclusivamente con fines de clasificación (Boscaini y col., 2004; Spitaler y col., 2007). Es por ello, que se decidió emplear la técnica de PTR-ToF-MS que permitió comparar el impacto de la matriz del vino en la dinámica de liberación del aroma. Para ello, se acopló una PTR-ToF-MS a un dispositivo de boca artificial, que permitía mimetizar determinados parámetros propios de la situación fisiológica (flujos, temperatura, agitación, presencia de saliva) eliminando la variabilidad interindividual y otros condicionantes (tipo ético, disponibilidad del panel, etc.) derivados del empleo de panelistas (**Publicación 4**). Para comprobar el efecto de la matriz vínica, de nuevo empleamos el mismo tipo de vinos de los trabajos anteriores (blanco, espumoso, tinto joven, tinto crianza y dulce) que fueron desaromatizados y reconstituidos a la misma concentración de etanol (12 %). Los vinos se aromatizaron con una mezcla de ocho compuestos volátiles, que fueron seleccionados por representar distintas familias químicas (diferentes propiedades físico-químicas), y en segundo lugar por su adecuada señal en la PTR-ToF-MS (baja fragmentación) que permitía emplearlos como moléculas target para estudiar su comportamiento en las distintas matrices vínicas en estudio.

Los resultados indicaron, contrariamente a lo observado en la **Publicación 1** (realizada en condiciones estáticas), que la mayor retención de aroma se observó en la matriz del vino dulce (alto contenido en azúcares y compuestos nitrogenados) y la mayor en vinos tintos. El proceso de elaboración del vino dulce (proceso de envejecimiento sobre lías), supone un elevado contenido en polisacáridos y en manoproteínas de levadura (Charpentier y Feuillat, 1993; Martínez-Rodríguez y Polo, 2000) que se ha comprobado tienen capacidad de unión de compuestos volátiles (Langorieux y Crouzet, 1997; Chalier y col., 2007). Por otra parte, la elevada viscosidad determinada en el vino dulce reconstituido en presencia de saliva, en comparación con el resto de matrices vínicas ensayadas pudo también estar relacionada con la menor liberación de aroma observada en este vino durante los primeros 30 segundos de monitorización, lo que indicaría una retención en los primeros momentos de la

liberación en boca (Roberts y Acree., 1996). Además, los vinos tintos fueron las matrices que liberaron más cantidad de aroma, y mostraron los valores más altos de todos los parámetros dinámicos extraídos de la curva de liberación (I_{max}, AUC, slope). La presencia de grandes concentraciones de polifenoles y polisacáridos en los vinos tintos de este estudio, junto con la presencia de las proteínas salivares, podría causar la formación de los macrocomplejos anteriormente comentados que actuarían como un reservorio de moléculas de aroma listas para ser liberadas en condiciones dinámicas, lo que explicaría la mayor liberación de aroma en vinos tintos. Además, y pese a que el contenido en polifenoles totales fue similar en el vino tinto joven (1647.98 mg/L) y en el vino tinto crianza (1672.62 mg/L), la liberación de aroma fue menor en el último, lo que pudo ser debido a diferencias en el estado y tipo de polimerización de los polifenoles.

Los resultados de este estudio eran muy interesantes ya que coincidían con los resultados obtenidos *in vivo* empleando el sistema de atrapamiento retronasal (**Publicación 3**). Pese a que en ambos experimentos se monitorizaron compuestos de aroma diferentes (con distintas propiedades físico-químicas), estos resultados ponen de manifiesto que el empleo de condiciones dinámicas para evaluar el impacto de ingredientes de un alimento, o en este caso de la matriz vínica, parecen las más adecuadas para monitorizar el aroma simulando condiciones reales consumo. Por tanto, además de corroborar la importancia de la matriz vínica a la hora de modular la cantidad y el tipo de aroma liberado en condiciones dinámicas más cercanas a las del consumo, estaba de acuerdo con estudios previos de la literatura que indican que la aproximación metodológica para el estudio de las interacciones entre los componentes del alimento y las moléculas aromáticas (métodos estáticos *vs* métodos dinámicos) puede influir en la naturaleza del efecto observado (Aznar y col., 2004 *vs* Tsachacki y col., 2005, Clark y col., 2011).

Además de los flujos de aire (capacidad respiratoria), durante el consumo de alimentos líquidos, hay otros parámetros relacionados con la fisiología oral, que se han descrito podrían afectar la composición del aroma retronasal. Es por ello, que durante la presente Tesis Doctoral se decidió evaluar el impacto de algunos de ellos (saliva, interacción con las mucosas orales, microbiota de la cavidad oral), considerando también la composición no volátil del vino.

La saliva es uno de los factores relacionados con la fisiología de la cavidad oral que más se ha estudiado, ya que durante el consumo de alimentos, no se perciben solamente las características propias del alimento, sino la sensación resultante de la mezcla del alimento con la saliva (Neyraud 2014). Está descrito que la presencia de saliva puede afectar el aroma retronasal por efecto de dilución, por interacciones entre compuestos de aroma y constituyentes de la saliva (proteínas), por su actividad enzimática o por su capacidad amortiguadora de pH, entre otras (Spielman 1990; Odake y col., 1998). En el caso del vino, el papel de la saliva más investigado está relacionado con la sensación de astringencia y sin embargo, hay muy pocos estudios dirigidos a comprender su influencia en la liberación de aroma, y los pocos resultados que existen, son a su vez contradictorios (Mitropoulou y col, 2011; Genovese y col., 2009). Esto puede ser debido, a diferencias en el protocolo experimental desarrollado (empleo de condiciones estáticas *vs* a dinámicas, distintos tipos de saliva, distinta concentración de etanol, etc.).

Por tanto, para dilucidar el papel que ejerce la saliva en la liberación de aroma, se optó por emplear dos modelos *in vitro* (en condiciones estáticas y dinámicas), ya que este tipo de estudios se hace inviable en condiciones *in vivo*. No obstante, durante el estudio se empleó saliva humana colectada de individuos sanos (n = 20) (**Publicación 5**).

En una primera etapa de trabajo se optimizaron las condiciones de un método de análisis en estático basado en HS-SPME y después se aplicó el método a dos matrices vínicas de composición muy diferente, sobre todo respecto al contenido de polifenoles totales (269.95 mg L^{-1} vino blanco y 1647.98 mg L^{-1} vino tinto), que como hemos visto en los anteriores trabajos parece que es un importante parámetro composicional de los vinos que afecta la liberación del aroma. Para este estudio, los vinos habían sido desaromatizados, reconstituidos y dopados con una solución de 45 aromas representativos del perfil volátil del vino, y el contenido de etanol se mantuvo constante (12 % de etanol). Posteriormente, se realizó el mismo procedimiento, pero en condiciones dinámicas, empleando para ello un dispositivo basado en un "bio-reactor" de saliva con temperatura, flujos y agitación controladas. Además empleando dos fibras de SPME diferentes se muestreó la liberación del aroma a dos tiempos diferentes "de consumo". El inicial (t = 0), que podría estar relacionado con la fase oral, en la que el vino generalmente frío se mezcla con la saliva lo que reduce la temperatura de la

cavidad oral (25.5 ºC) en comparación con la situación fisiológica (36 ºC). El tiempo final (t = 10) puede estar más relacionado con una situación post-oral (36 ºC), en la cual algunos volátiles podrían ser liberados de la muestra líquida y permanecer en la cavidad oral después del consumo a temperatura fisiológica (Buettner y col., 2001). Los vinos fueron incubados con saliva humana, saliva artificial o agua, lo que permitió extraer conclusiones sobre el mecanismo de acción de la saliva (interacción con proteínas, hidrólisis enzimática, etc.), eliminando el efecto de dilución.

Entre los principales resultados de este trabajo cabe destacar que se encontró un importante efecto de la saliva en la liberación de aroma. No obstante, este efecto fue diferente en función de la matriz vínica, del tipo de saliva y de la técnica empleada.

En general, se encontró un fuerte efecto de retención de la saliva en condiciones estáticas, lo que estaba de acuerdo con estudios previos que han sugerido que aunque las condiciones dinámicas simulan mejor la situación que se produce durante el consumo, las medidas estáticas son más adecuadas para determinar interacciones con buena precisión (Juteau y col., 2004). Sin embargo, en condiciones dinámicas, el efecto de retención por parte de la saliva fue menos evidente siendo mucho más marcado el efecto ejercido por la matriz vínica y por la temperatura de muestreo (fase oral *vs* fase post-oral). A pesar del menor impacto de la saliva observado en condiciones dinámicas, en una situación *in vivo* en la que la saliva está continuamente produciéndose e incorporándose a la cavidad oral, nos invita a pensar que el efecto puede ser mayor que el determinado en el presente estudio. Además, en las condiciones dinámicas y sobre todo en la fase oral (t = 0) un gran número de compuestos de aroma se liberaron más en los vinos incubados con saliva, lo que parecía contradecir el efecto de retención observado durante los experimentos en estático, y puso en evidencia una vez más la divergencia de resultados en ambas técnicas. Durante la fase de muestreo post-oral, se produjo un gran incremento de aroma liberado, lo que podría ser muy importante en la etapa tras el consumo relacionada con la persistencia de aroma.

Por otra parte, el diferente efecto observado en función del tipo de vino estuvo relacionado con la variación en la composición de la matriz vínica. En este sentido, se comprobó que los vinos tintos en presencia de saliva humana mostraron, en condiciones estáticas los valores más altos de retención de compuestos de aroma. La explicación que se postula para explicar este efecto es la posible la formación y presencia de los

anteriormente mencionados macrocomplejos (proteína de la saliva – polifenol – carbohidrato del vino). De hecho, la mayoría de los compuestos más retenidos fueron los más hidrofóbicos (log *P* > 2), lo que parece confirmar esta hipótesis, que se ha ido observando en situación dinámica (*in vivo* o *in vitro*) a lo largo de las **Publicaciones 3 y 4**.

Además, se encontraron diferentes efectos en función del tipo de saliva ensayada, lo que pudo ser debido a la diferente composición de las salivas (humana *vs* artificial). Una de las proteínas presentes en la saliva que se ha comprobado puede interaccionar con compuestos del aroma es la mucina (Friel y Taylor, 2001; van Ruth y col., 2001). Sin embargo, en nuestro experimento, los vinos incubados con saliva artificial (que contenía únicamente esta proteína pero una cantidad muy superior que la determinada en la saliva humana) ejercieron un menor efecto de retención que los vinos incubados con saliva humana. Para explicar este hecho, habría que pensar en la presencia de otro tipo de compuestos presentes en la saliva humana y que no estaban en la saliva artificial. En este sentido, es importante señalar que la saliva humana puede contener otras proteínas, como las PRPs que pueden representar hasta un 70 % de las proteínas segregadas por la glándula parótida (Mese y Matsuo, 2007). Estas proteínas pueden interaccionar con taninos del vino formando agregados (Canon et al., 2013). Sin embargo, cabe la posibilidad de que no sólo los taninos, sino otros componentes de la matriz vínica podrían también estar involucrados en la formación de estos agregados, como los macrocomplejos anteriormente comentados (proteína de la saliva – polifenol – carbohidrato del vino). Estas macroestructuras podrían encapsular o interaccionar con los compuestos del aroma del vino, lo que explicaría su retención, como comprobamos en condiciones estáticas. Además se comprobó que la formación de estos agregados no producía cambios en la viscosidad de la solución.

Por otra parte, en la bibliografía se ha descrito el posible papel enzimático de la saliva sobre compuestos del aroma. En la saliva humana se han descrito unos 30 enzimas (que incluyen amilasa, invertasa, maltasa, anhidrasa carbónica, ureasa, oxidasa, catalasa, enzimas proteolíticas, lipasa, fosfatasa, lisozima, e hialuronidasa) y recientemente se ha demostrado la acción de alguna de ellas sobre compuestos de aroma en soluciones acuosas (Buettner 2002b,c), o ha sido sospechada en vino (Genovese y col., 2009), o vino de palma (Lasekan 2013). Se ha sugerido que algunas enzimas esterolíticas presentes en la saliva como carboxilesterasas (Hussein y col., 1983;

Buettner 2002b) pueden degradar ésteres a sus ácidos correspondientes. En el presente estudio, se observó una disminución de ésteres en presencia de saliva humana, sin embargo, este efecto pareció ser debido a la interacción de estos compuestos con los macrocomplejos (polifenol-proteína-carbohidrato) anteriormente comentados, ya que no se observó el aumento de ácidos correspondientes. Otro grupo de compuestos susceptibles de ser hidrolizado por enzimas salivares son los aldehídos y pese a que se observó una disminución significativa de algunos de ellos, como el furfural, no se distinguió el aumento del correspondiente alcohol (furfuril alcohol). Por otra parte, se observó un aumento de otros compuestos, como los alcoholes terpénicos, que se pueden originar *de novo* a partir de precursores de aroma. Los precursores glicosídicos del aroma son compuestos inodoros que se encuentran de forma natural en las uvas y que representan un reservorio natural de moléculas potencialmente odorantes en el vino. Estos precursores podían haber estado presentes en las matrices desodorizadas e hidrolizarse en presencia de las enzimas salivares liberando las correspondientes agliconas volátiles. Sin embargo, las condiciones experimentales del presente estudio, no permitieron confirmar este efecto. Es importante tener en cuenta, que el proceso de estandarización de saliva humana empleado en este estudio (centrifugación, filtración, empleo de azida sódica, congelación), podría haber afectado los niveles de algunas proteínas y/o enzimas (Schipper y col., 2007).

Otro de los factores oro-fisiológicos que podrían tener relevancia en la percepción del aroma del vino, y que hasta el momento apenas han sido estudiados, son las mucosas oral y faríngea. Estas mucosas podrían interaccionar con las moléculas odorantes permitiendo aumentar el tiempo de residencia de compuestos de aroma en la cavidad oral y actuar como reservorios de moléculas odorantes que se liberarían tras el consumo contribuyendo al fenómeno de persistencia del aroma ("afterodour"), lo que está muy relacionado con la calidad de alimentos como el vino.

Para comprobar el papel de la mucosa oral se ha optimizado un método *in vivo* basado en el procedimiento SOOM (spit off odorant measurement)-GC/MS, mientras que la capacidad de liberación del aroma adsorbido a la mucosa oral se monitorizó mediante un método optimizado en el laboratorio basado en el análisis Intraoral-SPME-GC-MS. Para ello, los panelistas se enjuagaron la cavidad oral con un vino blanco aromatizado con 6 odorantes de grado alimentario y tras un tiempo determinado, lo expectoraron. Este procedimiento permitió calcular el grado de retención de los

compuestos monitorizados en la cavidad bucal que llegó hasta un 50 % para algunos de ellos, lo que puso de manifiesto la importancia de la mucosa oral en la retención de compuestos del aroma del vino. No obstante, esta retención fue dependiente de las características físico-químicas de los compuestos evaluados, mostrando en general, los compuestos más hidrofóbicos una mayor retención, lo que indicó la existencia de posibles enlaces hidrofóbicos con las proteínas de la saliva, posiblemente en la llamada película salivar de la mucosa, que es una película formada por proteínas de la saliva que se forma sobre la superficie de la mucosa con finalidad de lubricación y protección (Bradway y col., 1992; Humphrey y Williamson, 2001; Carpenter 2013). Además, la cinética de liberación de los compuestos adsorbidos a la mucosa (Intraoral-SPME-GC/MS) confirmó que los aromas monitorizados se siguen liberando tiempo después del consumo (> 5 min) lo que podría estar relacionado con la mayor o menor persistencia de notas aromáticas de los vinos. En general, los compuestos más volátiles característicos por aportar notas frutales (acetato de isoamilo, hexanoato de etilo) desaparecieron antes que los menos volátiles que aportan notas florales (linalool, β-ionona, β-feniletanol) o especiadas (guaiacol), lo que estuvo de acuerdo con los resultados de un estudio sensorial previo realizado por Goodstein y col. (2014). Además, se comprobó el efecto de la matriz no volátil en la retención y posterior liberación de los compuestos del aroma mediante el estudio de regresiones lineales calculadas en dos vinos (blanco y tinto) aromatizados con concentraciones crecientes de los seis compuestos anteriormente mencionados. En general, el vino tinto mostró unos valores de liberación menores que el vino blanco, a pesar de que partir del mismo nivel de compuestos de aroma. Esto indicó una mayor interacción de los compuestos del aroma cuando se incorporan en una matriz tinta, que se traduce en menor liberación. En las condiciones experimentales empleadas, la boca humana puede considerarse un reactor cerrado por dos barreras, la anterior formada por los labios y la posterior formada por la barrera entre el velo del paladar y la lengua. Esta configuración anatómica hace que durante el tiempo de muestreo, el interior de la boca es un sistema estático (no hay flujos de aire). Aunque la magnitud del efecto de retención dependía de las características de cada compuesto, este resultado confirma de nuevo, la hipótesis manejada a lo largo de la Tesis, mediante la cual, la formación de complejos entre proteínas (en este caso, con la película salivar de la mucosa), polifenoles y carbohidratos del vino puede resultar en un depósito de compuestos de aroma, que en condiciones estáticas (como las del presente experimento y **Publicación 5**) provoca una

mayor retención de aroma, pero que en condiciones dinámicas producirá una mayor cantidad de aroma liberado (**Publicación 3, 4 y 5**).

Por último, la cavidad oral es un hábitat muy adecuado para el crecimiento microbiano. La presencia de microrganismos en la cavidad oral podría resultar en una metabolización de los constituyentes alimentarios, provocando una disminución de determinados compuestos y la formación otros de nuevos que podrían modificar la percepción de aroma durante el consumo. Recientemente, Starkenman y col. (2008) comprobaron las bacterias anaerobias presentes en la cavidad oral pueden hidrolizar precursores cisteínicos de aroma presentes en algunas especies vegetales. Aunque en este estudio no se pudieron determinar las agliconas volátiles liberadas, debido a que el límite de detección de la técnica empleada era demasiado alto, los autores determinaron el efecto sensorial en un estudio *in vivo*. Además en un trabajo reciente Mayr y col. (2014) han sugerido que la degradación *in vivo* de precursores no odorantes de compuestos fenólicos volátiles podría ser debida a la acción de la microbiota oral. Pese a estos incipientes trabajos, hasta el momento el papel de la microbiota oral en la generación de compuestos del aroma a partir de precursores de la uva se desconoce. Por otra parte, a menudo se ha sugerido el potencial de las enzimas salivares en la metabolización de compuestos de aroma, aunque este efecto sólo ha podido ser comprobado en soluciones acuosas (Buettner 2002b,c).

Era, por tanto, interesante evaluar la capacidad de las enzimas salivares y de la microbiota de la cavidad oral en la metabolización de compuestos potencialmente odorantes del vino. Para este trabajo se decidió trabajar con precursores glicosídicos de aroma, ya que son un grupo de compuestos que se encuentran en altas concentraciones en las uvas y suponen un importante reservorio de aroma cuya hidrólisis genera agliconas volátiles con bajos umbrales de olfacción que pueden aportar notas aromáticas notas aromáticas positivas a los vinos.

Sin embargo, estas moléculas no están disponibles comercialmente por lo que previamente fue necesario disponer de técnicas de aislamiento que proporcionaran elevados rendimientos de extracción de este tipo de compuestos tanto de uvas como de orujos de uvas. La técnica convencional de extracción líquido-líquido ha sido ampliamente utilizada (Hernández-Orte y col., 2009) y además, se evaluó la posibilidad de emplear otra técnica de extracción (PLE) con solventes GRAS que ha demostrado ser

eficaz para la extracción de diferentes fitoquímicos de plantas. La comparación de ambas técnicas se llevó a cabo empleando como materia prima subproductos de vinificación, cuya acumulación supone un importante problema medioambiental. Estos resultados dieron lugar a la **Publicación 7.** Gracias a la aplicación de estas técnicas, se comprobó por una parte, que estos residuos (fundamentalmente hollejos de uva) contienen precursores de aroma, cuya hidrólisis libera compuestos odorantes, con lo que podrían ser interesantes como materia prima para diferentes aplicaciones industriales. Además se evaluó la eficacia de dos técnicas de aislamiento y se comprobó que, en general, las agliconas volátiles (compuestos terpénicos, fenoles volátiles y alcoholes) se extrajeron con mayor eficiencia utilizando la extracción con solventes presurizados, la cual además proporcionaba menores tiempos de extracción (30 min frente a 48 horas). Por lo tanto, los extractos obtenidos con las diferentes metodologías se utilizaron para evaluar el papel de microbiota bacteriana oral en la generación de compuestos de aroma. Sin embargo, en experimentos previos se observó que el extracto obtenido mediante PLE inhibía el crecimiento de las bacterias orales lo que podía deberse al alto contenido en polifenoles favorecido por las condiciones de extracción empleadas, por lo que finalmente, en el siguiente estudio dirigido a evaluar la acción de compuesto aromáticos por la microbiota oral, se empleó solo el extracto obtenido por maceración estática (**Publicación 8)**.

Para ello, en un primer experimento *in vitro*, se evaluó la capacidad de hidrólisis de precursores glicosídicos por parte de nueve bacterias que pueden encontrarse de manera natural en la boca de individuos sanos (*Streptococcus sanguinis*, *Streptococcus oralis*, *Actinomyces naeslundii*, *Streptococcus mutans*, *Veillonella dispar*, *Fusobacterium. nucleatum*, *Streptococcus aureus* y *Enterococcus faecalis*). Las agliconas odorantes fueron extraídas y analizadas por HS-SPME-GC/MS y los resultados mostraron la capacidad de estos microorganismos para hidrolizar precursores glicosídicos de aroma liberando los correspondientes terpenos, C6-alcoholes, derivados bencénicos, etc., que son moléculas caracterizadas por presentar bajos umbrales de olfacción y notas aromáticas positivas en el vino, lo que podría influir la percepción del aroma durante el consumo de vino. Sin embargo, se comprobó que la transformación de glicósidos fue bacteria-dependiente, y así bacterias como *A. naeslundii* y *S. mutans* fueron las mayores productoras, mientras que otras como *G. adiascens*, *V. dispar* y *F. nucleatum* fueron las menores productoras. Para elucidar la significancia de estos

resultados es interesante entender la presencia de estas bacterias en la cavidad oral. La cavidad oral es un sistema dinámico y heterogéneo que está compuesto de diferentes microambientes. Las superficies de la boca (como los dientes) a menudo son colonizadas por biofilms bacterianos (conjunto de bacterias aerobias y anaerobias), que son frecuentemente eliminados, por descamación de células epiteliales o por movimientos mecánicos como el cepillado de dientes (Jakubovics y Kloenbrander, 2010). Sin embargo, las bacterias orales se adaptan fácilmente y son capaces de recolonizar las superficies en minutos. Las primeras bacterias en colonizar las superficies orales son streptococos y actinomices (Rickard y col., 2003; Li y col., 2004), y por lo tanto, estos microorganismos son los predominantes en las primeras etapas de la formación del biofilm dental (4-8 h) (Li y col., 2004). Este hecho tiene especial relevancia porque estos microorganismos (*A. naeslundii, S. mutans*) fueron los que mostraron mayor capacidad de liberar agliconas aromáticas. Este ha sido el primer estudio que ha demostrado la capacidad de varias bacterias representativas de la cavidad oral para hidrolizar precursores de aroma glicosídicos.

Posteriormente, en un intento por entender el papel que el conjunto de la microbiota oral humana podría desempeñar en la formación de compuestos odorantes a partir de precursores glicosídicos de la uva, se empleó una aproximación *ex vivo*, en la que saliva fresca procedente de tres individuos (n = 3) fue incubada en condiciones aerobias y anaerobias, en un medio de cultivo óptimo que se ha comprobado es capaz de mantener el crecimiento de comunidades bacterianas diversas con un perfil similar al encontrado en la microbiota de la saliva de partida (Tian y col., 2010). Las muestras se incubaron con el extracto de precursores a diferentes tiempos (0, 2, 24, 72 h). El mismo procedimiento se llevó a cabo utilizando también saliva estéril (sin microorganismos) y no enzimática (sin microorganismos, ni enzimas), para evaluar el papel de las enzimas de la saliva.

De acuerdo con los resultados anteriormente descritos, los microorganismos crecidos tanto en condiciones aerobias como anaerobias fueron capaces de hidrolizar los precursores. Incluso más interesante fue el hecho de que en los controles (sin microorganismos y sin microorganismos ni enzimas) no se aislaron agliconas aromáticas, confirmando que la capacidad hidrolítica fue únicamente debida a la presencia de bacterias vivas, descartándose la acción enzimática de la saliva, al menos sobre precursores glicosídicos.

Por otra parte, es conocida la gran variedad interindividual en la composición de la microbiota oral humana (Bik y col., 2010). Para evaluar esta posible fuente de variabilidad, se llevó a cabo un segundo experimento *ex vivo* dirigido a evaluar las consecuencias que esta variable pudiera tener en la hidrólisis de precursores glicosídicos. Para ello, se desarrolló el mismo procedimiento experimental anteriormente descrito pero, en este caso, las muestras de saliva aisladas de tres individuos sanos fueron tratadas de forma independiente. Los resultados permitieron demostrar una gran variación interindividual en la capacidad de liberación de agliconas aromáticas, tanto en el caso del cultivo aerobio como en el anaerobio, lo que estaba de acuerdo con estudios previos (Walle y col., 2005). Esta conclusión, es muy importante porque indicaba que la hidrólisis oral y por tanto la liberación de aroma puede ser diferente para cada individuo en función de la composición de su microbiota oral, lo que podría ser importante a la hora de entender las diferencias entre individuos en la percepción de aroma durante el consumo. De hecho, se comprobó que estas diferencias están más relacionadas con diferencias cualitativas (tipo de bacteria) que cuantitativas (cantidad de bacterias presentes en la saliva).

Este resultado, además de novedoso, es de gran interés, ya que muchas de las agliconas liberadas a partir de los precursores presentan umbrales de detección muy bajos, lo que significa que aún en muy bajas concentraciones su presencia podría tener un gran impacto en la percepción de aroma, como previamente Starkenman y col. (2008) y Mayr y col. (2014) demostraron en el caso de percusores cisteínicos y fenólicos. Por otro lado, aunque los resultados obtenidos implicaron tiempos de incubación largos, es importante tener en cuenta que la mucosa oral podría retener este tipo de compuestos (Buettner y col., 2001), incrementando su tiempo de residencia en la boca y haciéndoles más susceptibles a los factores fisiológicos, como la acción de la microbiota oral.

Todos estos resultados parecen confirmar que el modo en que los compuestos del aroma se liberan en la cavidad oral durante el consumo y su capacidad de interacción con los distintos fluidos y tejidos del organismo podría modificar y provocar cambios en la cantidad y el tipo de compuestos que pueden interaccionar con los órganos olfativos. El desarrollo de este tipo de técnicas de análisis instrumental dinámicas e incorporando la presencia de diferentes parámetros orofisiológicos permiten entender la situación fisiológicas durante el consumo, y por tanto, están más

relacionadas con la percepción retronasal, que las técnicas clásicas de análisis centradas exclusivamente en el alimento. Sin embargo, es importante recordar que en la percepción del aroma también intervienen otras experiencias sensoriales, como son el sabor, textura y color, así como otros factores socioculturales, psicológicos, cognitivos, religiosos, etc., que hacen que la percepción del aroma de un mismo alimento pueda ser muy diferente de un individuo a otro. Todos estos factores y la interacción entre unos y otros son piezas claves del puzle necesarias para entender la percepción del aroma durante el consumo de los alimentos.

Conclusiones

6. CONCLUSIONES

1. La composición de la matriz no volátil del vino afecta fuertemente la volatilidad de los compuestos de aroma en condiciones estáticas (HS-SPME-GC/MS). Principalmente se observó un efecto de retención del aroma debido a la presencia de macromoléculas (como las glicoproteínas), aunque en los vinos con un alto contenido en compuestos de pequeño tamaño molecular (mono y disacáridos, aminoácidos libres) también se observó un efecto "salting out". La familia química y en particular las características físico-químicas (volatilidad y log P), influyeron fuertemente en este comportamiento.

2. El sistema de atrapamiento de aroma retronasal (RATD) basado en el uso de trampas Tenax como adsorbente polimérico desarrollado en este trabajo, junto con la optimización de un protocolo de consumo, permite de una manera simple, conveniente y precisa, la evaluación del impacto de los componentes de la matriz vínica o de ingredientes en bebidas a base de vino en la liberación de aroma en una situación real de consumo.

3. Se ha demostrado que diferencias interindividuales asociadas a la fisiología oral durante el consumo (capacidad respiratoria) afectan la liberación de aroma *in vivo*, lo que permite una agrupación de los panelistas entre altos y bajos liberadores de aroma.

4. Durante el consumo de los vinos de este estudio, los vinos tintos (joven y crianza) produjeron una mayor liberación de aroma comparados con los vinos blancos (blanco y espumosos) y dulce. Los polifenoles totales fueron los compuestos más correlacionados entre los componentes de la matriz vínica y el aroma liberado por los panelistas. Este efecto pudo ser debido a la implicación de estas macromoléculas en la formación de un recubrimiento en la mucosa oral o faríngea tras la deglución, lo que podría aumentar el área de contacto entre el aire exhalado y el vino y/o a la formación de complejos de polifenoles/aroma en la superficie del recubrimiento que actuaría como un reservorio de moléculas de aroma listas para ser liberadas por los flujos respiratorios.

5. El empleo de una PTR-ToF-MS acoplada a una boca artificial permite la monitorización de la liberación de aroma en tiempo real y el cálculo de los parámetros dinámicos de liberación de aroma (Imax, AUC, pendiente). Gracias a esta técnica se ha confirmado la gran influencia de la matriz no volátil en la liberación de aroma y la

implicación de los polifenoles en la mayor liberación de aroma determinado en los vinos tintos.

6. La saliva ejerce un efecto importante en la liberación de aroma del vino. Este efecto ha sido probado en condiciones estáticas y dinámicas. Sin embargo, la primera aproximación es más adecuada para evaluar interacciones entre la matriz-aroma-saliva, mientras que la segunda es más apropiada para simular la situación de consumo. El efecto de la saliva es dependiente de la composición de la matriz no volátil. Los vinos tintos mostraron una menor liberación de aroma que pareció ser debido a la interacción de los compuestos de aroma con unos complejos formados por proteínas de la saliva (PRPs) y polifenoles y polisacáridos del vino. Estas interacciones fueron más importantes en el caso de compuestos hidrofóbicos con un elevado log *P*.

7. De los estudios *in vivo*, se ha confirmado que la mucosa oral es capaz de retener compuestos de aroma del vino y liberarlos posteriormente. Esta capacidad depende de algunas propiedades físico-químicas de los compuestos aromáticos (log *P* y volatilidad). Los odorantes adsorbidos a la mucosa oral pueden ser liberados de manera diferente dependiendo de la composición de la matriz del vino. En general, los vinos tintos liberan menos aroma que los vinos blancos, lo que podría estar relacionado con una mayor adsorción de los volátiles, debida a la formación de complejos de aroma-polifenoles-glicoproteínas de la saliva en la superficie de la película de mucosa en la cavidad oral.

8. La microbiota oral (cepas bacterianas o el conjunto de la microbiota aislada a partir de individuos sanos) es capaz de producir compuestos odorantes a partir de precursores no odorantes de la uva y del vino. Esta capacidad es cepa-dependiente y está relacionada con sus necesidades de crecimiento (anaerobiosis, aerobiosis). Se ha observado una alta variación en la generación de agliconas odorantes entre individuos, que parece estar más vinculada a diferencias cualitativas en la composición de la microbiota que a diferencias cuantitativas.

Conclusions

6. CONCLUSIONS

1. The non-volatile wine matrix composition strongly influences the volatility of wine aroma compounds in static conditions (HS-SPME-GC/MS) mainly by a retention effect produced by wine macromolecules (e.g. glycoproteins); although a "salting out" effect was observed in wines with a high concentration of small molecules (mono and disaccharides, free amino acids). The aroma chemical class, in particular its physicochemical properties (volatility and log *P* value), strongly influences this behaviour.

2. The retronasal aroma trapping device (RATD) using Tenax as adsorbent polymer developed in this work together with an optimised consumption procedure, allows in a simple, convenient, and precise way, the evaluation of the impact of wine matrix components or ingredients in wine base beverages, on aroma release during real drinking conditions.

3. It has been shown that interindividual differences associated to oral physiology during wine consumption, such as breathing capacity, affects *in vivo* aroma release during drinking, which allowed clustering the panelists between higher and lower aroma releasers.

4. During the consumption of the wines of this study, red wines (young and aged) produced a higher aroma release compared with white (cava and white) and sweet wines. The highest correlation among wine matrix components and aroma release was observed for the total polyphenol content. This effect could be due to the involvement of these compounds on the formation of a wine coating after swallowing which might increase the contact area between exhaled air and product and/or because of the formation of polyphenol-aroma complexes on the surface coating acting as a reservoir of aroma molecules ready to be released by the expiration flows.

5. The use of PTR-ToF-MS coupled to an artificial mouth allows monitoring aroma release at real time by calculating the dynamic aroma release parameters (Imax, AUC, slope). By using this technique it has been confirmed the great influence of wine non volatile matrix composition on aroma release and the involvement of total polyphenols on the higher aroma release determined in red wines.

6. Saliva has an important effect on aroma release from wine. This effect has been proven in static and dynamic conditions. Nonetheless, the former technique is better suited to evaluate aroma-saliva-matrix interactions, while the latter is more appropriate to simulate a consumption situation. Saliva effect is dependent on wine matrix composition. Red wines shows a lower aroma release which seem to be due to the interactions of aroma compounds with complexes formed by saliva proteins (proline rich proteins, PRPs) and wine polyphenols and polysaccharides. These aroma interactions are stronger in the case of hydrophobic compounds with a high log P value.

7. From *in vivo* studies, it has been confirmed that Oral mucosa is able to retain wine aroma compounds and further release them. This capacity is dependent on some physicochemical properties of the aroma compounds (log *P* and volatility). Odorants adsorbed to oral mucosa can be differently released depending on wine matrix composition. In general, red wines released lower aroma than white wines, which could be related to a higher adsorption of odorants because of the formation of aroma-polyphenol- saliva glycoproteins complexes in the surface of the mucus pellicle in the oral cavity.

8. Oral microbiota (individual strains or the whole microbiota isolated from health individuals) is able to produce odorant compounds from odorless grape and wine glycosides. This capacity is bacteria-dependent and related to their growth requirements (anaerobe, aerobe). A high variation in the aglycone generation is observed among individuals, which seem to be more linked to qualitative differences in microbiota composition than to quantitative differences.

Bibliografia

7. BIBLIOGRAFÍA

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology*, *43* (11), 5721-5732.

Acree, T. E., Barnard, J., & Cunningham, D. G. (1984). A procedure for the sensory analysis of gas chromatographic effluents. *Food Chemistry*, *14* (4), 273-286.

Andújar-Ortiz, I., Moreno-Arribas, M. V., Martín-Álvarez, P. J., & Pozo-Bayón, M. A. (2009). Analytical performance of three commonly used extraction methods for the gas chromatography-mass spectrometry analysis of wine volatile compounds. *Journal of Chromatography A*, *1216* (43), 7351-7357.

Aprea, E., Biasioli, F., Mark, T. D., & Gasperi, F. (2007). PTR-MS study of esters in water and water/ethanol solutions: fragmentation patterns and partition coefficients. *International Journal of Mass Spectrometry*, *262* (1-2), 114-121.

Aronson, J., & Ebeler, S. E. (2004). Effect of polyphenol compounds on the headspace volatility of flavors. *American Journal of Enology and Viticulture*, *55* (1), 13-21.

Arvisenet, G., Billy, L., Poinot, P., Vigneau, E., Bertrand, D., & Prost, C. (2008). Effect of apple particle state on the release of volatile compounds in a new artificial mouth device. *Journal of Agricultural and Food Chemistry*, *56* (9), 3245-3253.

Atanasova, B., Thomas-Danguin, T., Chabanet, C., Langlois, D., Nicklaus, S., & Etievant, P. (2005). Perceptual interactions in odour mixtures: Odour quality in binary mixtures of woody and fruity wine odorants. *Chemical Senses*, *30* (3), 209-217.

Athès, V., Peña Y Lillo, M., Bernard, C., Pérez-Correa, R., & Souchon, I. (2004). Comparison of experimental methods for measuring infinite dilution volatilities of aroma compounds in water/ethanol mixtures. *Journal of Agricultural and Food Chemistry*, *52* (7), 2021-2027.

Aubry, V., Etievant, P., Sauvageot, F., & Issanchou, S. (1999). Sensory analysis of Burgundy Pinot noir wines: A comparison of orthonasal and retronasal profiling. *Journal of Sensory Studies*, *14* (1), 97-117.

Avison, S. J. (2013). Real-Time Flavor Analysis: Optimization of a Proton-Transfer-Mass Spectrometer and Comparison with an Atmospheric Pressure Chemical Ionization Mass Spectrometer with an MS-Nose Interface. *Journal of Agricultural and Food Chemistry*, *61* (9), 2070-2076.

Aznar, M., Tsachaki, M., Linforth, R., Ferreira, V., & Taylor, A. (2004). Headspace analysis of volatile organic compounds from ethanolic systems by direct APCI-MS. *International Journal of Mass Spectrometry*, *239* (1), 17-25.

B

Bayonove, C., Baumes, R., Crouzet, J., & Günata, Z. (2000). En: C. Flanzy, *Enología: Fundamentos Científicos y Tecnológicos* (p. 137-176). Madrid: AMV and Mundi-Prensa Editions.

Benjamin, O., Silcock, P., Beauchamp, J., Buettner, A., & Everett, D. W. (2012). Tongue Pressure and Oral Conditions Affect Volatile Release from Liquid Systems in a Model Mouth. *Journal of Agricultural and Food Chemistry*, *60* (39), 9918-9927.

Bennick, A. (1982). Salivary proline-rich proteins. *Molecular and Cellular Biochemistry*, *45* (2), 83-99.

Bik, E. M., Long, C. D., Armitage, G. C., Loomer, P., Emerson, J., Mongodin, E. F., Nelson, K. E., Gill, S. R., Fraser-Liggett, C. M., & Relman, D. A. (2010). Bacterial diversity in the oral cavity of 10 healthy individuals. *Isme Journal*, *4*(8), 962-974.

Blake, R. S., Whyte, C., Hughes, C. O., Ellis, A. M., & Monks, P. S. (2004). Demonstration of proton-transfer reaction time-of-flight mass spectrometry for real-time analysis of trace volatile organic compounds. *Anal. Chem*, *76* (13), 3841-3845.

Blee, N., Linforth, R., Yang, N., Brown, K., & Taylor, A. (2011). Variation in aroma release between panellists consuming different types of confectionary. *Flavour and Fragrance Journal*, *26* (3), 186-191.

Boelrijk A. E., Smith G., Weel K. G. (2006). En: A. Voilley and P. Etievant, *Flavour in Food* (p. 260-279). New York: CRC Press.

Boland, A. B., Buhr, K., Giannouli, P., & van Ruth, S. M. (2004). Influence of gelatin, starch, pectin and artificial saliva on the release of 11 flavour compounds from model gel systems. *Food Chemistry*, *86* (3), 401-411.

Boscaini, E., Mikoviny, T., Wisthaler, A., von Hartungen, E., & Mark, T. D. (2004). Characterization of wine with PTR-MS. *International Journal of Mass Spectrometry*, *239* (2-3), 215-219.

Bourdiol, P., Mishellany-Dutour, A., Peyron, M.-A., & Woda, A. (2013). Contributory role of the tongue and mandible in modulating the in-mouth air cavity at rest. *Clinical Oral Investigations*, *17* (9), 2025-2032.

Boze, H., Marlin, T., Durand, D., Perez, J., Vernhet, A., Canon, F., Sarni-Manchado, P., Cheynier, V., & Cabane, B. (2010). Proline-Rich Salivary Proteins Have Extended Conformations. *Biophysical Journal*, *99*(2), 656-665.

Bradway, S. D., Bergey, E. J., Scannapieco, F. A., Ramasubbu, N., Zawacki, S., & Levine, M. J. (1992). Formation of salivary-mucosal pellicle - the role of transglutaminase. *Biochemical Journal*, *284*, 557-564.

Brauss, M. S., Linforth, R. S. T., & Taylor, A. J. (1998). Effect of variety, time of eating, and fruit-to-fruit variation on volatile release during eating of tomato fruits (Lycopersicon esculentum). *Journal of Agricultural and Food Chemistry*, *46* (6), 2287-2292.

Brauss, M. S., Linforth, R. S. T., Cayeux, I., Harvey, B., & Taylor, A. J. (1999). Altering the fat content affects flavor release in a model yogurt system. *Journal of Agricultural and Food Chemistry*, *47* (5), 2055-2059.

Buettner, A., & Schieberle, P. (2000). Exhaled odorant measurement (EXOM) — a new approach to quantify the degree of in-mouth release of food aroma compounds. *Lebensmittel-Wissenschaft und-Technologie*, *33* (8), 553-559.

Buettner, A., Beer, A., Hannig, C., & Settles, M. (2001). Observation of the swallowing process by application of videofluoroscopy and real-time magnetic resonance imaging-consequences for retronasal aroma stimulation. *Chemical Senses*, *26* (9), 1211-1219.

Buettner, A., Beer, A., Hannig, C., Settles, M., & Schieberle, P. (2002a). Physiological and analytical studies on flavor perception dynamics as induced by the eating and swallowing process. *Food Quality and Preference*, *13* (7-8), 497-504.

Buettner, A. (2002b). Influence of human salivary enzymes on odorant concentration changes occurring in vivo. 1. Esters and thiols. *Journal of Agricultural and Food Chemistry*, *50* (11), 3283-3289.

Buettner, A. (2002c). Influence of human saliva on odorant concentrations. 2. aldehydes, alcohols, 3-alkyl-2-methoxypyrazines, methoxyphenols, and 3-hydroxy-4,5-dimethyl-2(5H)-furanone. *Journal of Agricultural and Food Chemistry*, *50* (24), 7105-7110.

Buettner, A. (2004). Investigation of potent odorants and afterodor development in two Chardonnay wines using the buccal odor screening system (BOSS). *Journal of Agricultural and Food Chemistry*, *52* (8), 2339-2346.

Buettner, A., & Welle, F. (2004). Intra-oral detection of potent odorants using a modified stir-bar sorptive extraction system in combination with HRGC-O known as the buccal odor screening system (BOSS). *Flavour and Fragrance Journal*, *19* (6), 505-514.

Buettner, A., & Mestres, M. (2005). Investigation of the retronasal perception of strawberry aroma aftersmell depending on matrix composition. *Journal of Agricultural and Food Chemistry*, *53* (5), 1661-1669.

Buettner, A., Otto, S., Beerc, A., Mestres, M., Schieberle, P., & Hummel, T. (2008). Dynamics of retronasal aroma perception during consumption: Cross-linking on-line breath analysis with medico-analytical tools to elucidate a complex process. *Food Chemistry*, *108*, 1234-1246.

Buratti, S., Ballabio, D., Benedetti, S., & Cosio, M. S. (2007). Prediction of Italian red wine sensorial descriptors from electronic nose, electronic tongue and

spectrophotometric measurements by means of Genetic Algorithm regression models. *Food Chemistry*, *100* (1), 211-218.

Burdach, K. J., Kroeze, J. H. A., & Koster, E. P. (1984). Nasal, retronasal, and gustatory perception - an experimental comparison. *Perception & Psychophysics*, *36* (3), 205-208.

C

Camara, J. S., Alves, M. A., & Marques, J. C. (2006). Development of headspace solid-phase microextraction-gas chromatography-mass spectrometry methodology for analysis of terpenoids in Madeira wines. *Analytica Chimica Acta*, *555* (2), 191-200.

Campo, E., Ferreira, V., Escudero, A., Marques, J. C., & Cacho, J. (2006). Quantitative gas chromatography-olfactometry and chemical quantitative study of the aroma of four Madeira wines. *Analytica Chimica Acta*, *563*(1-2), 180-187.

Campo, E., Ferreira, V., Lopez, R., Escudero, A., & Cacho, J. (2006). Identification of three novel compounds in wine by means of a laboratory-constructed multidimensional gas chromatographic system. *Journal of Chromatography A*, *1122*(1-2), 202-208.

Campo, E., Cacho, J., & Ferreira, V. (2007). Solid phase extraction, multidimensional gas chromatography mass spectrometry determination of four novel aroma powerful ethyl esters. Assessment of their occurrence and importance in wine and other alcoholic beverages. *Journal of Chromatography A*, *1140*(1-2), 180-188.

Canon, F., Pate, F., Cheynier, V., Sarni-Manchado, P., Giuliani, A., Perez, J., Durand, D., Li, J., & Cabane, B. (2013). Aggregation of the Salivary Proline-Rich Protein IB5 in the Presence of the Tannin EgCG. *Langmuir*, *29*(6), 1926-1937.

Carpenter, G. H. (2013). The secretion, components and properties of saliva. *Annual Review of Food Science and Technology*, *4*, 267-276.

Clark, R., Linforth, R., Bealin-Kelly, F., & Hort, J. (2011). Effects of Ethanol, Carbonation and Hop Acids on Volatile Delivery in a Model Beer System. *Journal of the Institute of Brewing*, *117* (1), 74-81.

Coelho, E., Coimbra, M. A., Nogueira, J. M. F., & Rocha, S. M. (2009). Quantification approach for assessment of sparkling wine volatiles from different soils, ripening stages, and varieties by stir bar sorptive extraction with liquid desorption. *Analytica Chimica Acta*, *635* (2), 214-221.

Comuzzo, P., Tat, L., Tonizzo, A., & Battistutta, F. (2006). Yeast derivatives (extracts and autolysates) in winemaking: release of volatile compounds and effects on wine aroma volatility. *Food Chemistry*, *99* (2), 217-230.

Conner, J. M., Birkmyre, L., Paterson, A., & Piggott, J. R. (1998). Headspace concentrations of ethyl esters at different alcoholic strengths. *Journal of the Science of Food and Agriculture*, *77* (1), 121-126.

Chalier, P., Angot, B., Delteil, D., Doco, T., & Gunata, Z. (2007). Interactions between aroma compounds and whole mannoprotein isolated from Saccharomyces cerevisiae strains. *Food Chemistry*, *100* (1), 22-30.

Charles, M., Poinot, P., Texier, F., Arvisenet, G., Vigneau, E., Mehinagic, E., & Prost, C. (2013). The 'Mouth to Nose Merging System': A novel approach to study the impact of odour on other sensory perceptions. *Food Quality and Preference*, *28* (1), 264-270.

Chassagne, D., Ramirez, G., Lubbers, S., Charpentier, C., Feuillat, M., & Voilley, A. (2003). Sorption of aroma compounds to oak wood in wine ageing conditions. En: Flavour research at the down of the twenty-first century - Proceedings of the 10th Weurman flavour research symposium (p. 4). Presented at 10. Weurman flavour research symposium, Beaune, FRA (2003-06-24 - 2003-06-28). Paris, FRA : Lavoisier. http://prodinra.inra.fr/record/69004.

Chassagne, D., Guilloux-Benatier, M., Alexandre, H., & Voilley, A. (2005). Sorption of wine volatile phenols by yeast lees. *Food Chemistry*, *91* (1), 39-44.

Chauncey, H. H., Lionetti, F., Winer, R. A., & Lisanti, V. F. (1954). Enzymes of human saliva. 1.The determination, distribution, and origin of whole saliva enzymes. *Journal of Dental Research*, *33* (3), 321-334.

Culleré, L., Escudero, A., Pérez-Trujillo, J. P., Cacho, J., & Ferreira, V. (2008). 2-Methyl-3-(methyldithio)furan: a new odorant identified in different monovarietal red wines from the Canary Islands and aromatic profile of these wines. *Journal of Food Composition and Analysis*, *21*(8), 708-715.

D

d'Acampora Zellner, B., Dugo, P., Dugo, G., & Mondello, L. (2008). Gas chromatography-olfactometry in food flavour analysis. *Journal of Chromatography A*, *1186* (1-2), 123-143.

Dawes, C. (1981). The effects of exercise on protein and electrolyte secretion in parotid-saliva. *Journal of Physiology-London*, *320*, 139.

Delarue, J., & Giampaoli, P. (2006). Carbohydrate-flavour interactions. *Flavour in Food*(120), 208-228.

De Roos, K. B., & Wolswinkel, K. (1994). Non-equilibrium partition model for predicting flavour release in the mouth. En: H. Maarse, & D. G. van der Heij, *Trends in Flavour Research* (p. 15-32). Amsterdam: Elsevier Science.

De Roos, K. B. (2006). Modelling aroma interactions in food matrices. *Flavour in Food* (120), 229-259.

Deibler, K., Lavin, E., Linforth, R., Taylor, A. J., & Acree, T. E. (2001). Verification of a mouth simulator by in vivo measurements. *Journal Agricultural and Food Chemistry*, *49* (3), 1388–1393.

Delahunty, C., Piggott, J., Conner, J., & Paterson, A. (1996). Comparison of dynamic flavour release from hard cheeses and analysis of headspace volatiles from the mouth with flavour perception during consumption. *Journal of the Science of Food and Agriculture*, *71* (3), 273–281.

Deleris, I., Saint-Eve, A., Guo, Y., Lieben, P., Cypriani, M.-L., Jacquet, N., Brunerie, P., & Souchon, I. (2011). Impact of Swallowing on the Dynamics of Aroma Release and Perception during the Consumption of Alcoholic Beverages. *Chemical Senses*, *36* (8), 701-713.

Deleris, I., Saint-Eve, A., Semon, E., Guillemin, H., Guichard, E., Souchon, I., & Le Quere, J.-L. (2013). Comparison of direct mass spectrometry methods for the on-line analysis of volatile compounds in foods. *Journal of Mass Spectrometry*, *48* (5), 594-607.

Denker, M., Parat-Wilhelms, M., Drichelt, G., Paucke, J., Luger, A., Borcherding, K., Hoffmann, W., & Steinhart, H. (2006). Investigation of the retronasal flavour release during the consumption of coffee with additions of milk constituents by 'Oral Breath Sampling'. *Food Chemistry*, *98* (2), 201-208.

Denny, P., Hagen, F. K., Hardt, M., Liao, L., Yan, W., Arellanno, M., Bassilian, S., Bedi, G. S., Boontheung, P., Cociorva, D., Delahunty, C. M., Denny, T., y col., (2008). The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *Journal of Proteome Research*, *7*(5), 1994-2006.

Doyen, K., Carey, M., Linforth, R. S. T., Marin, M., & Taylor, A. J. (2001). Volatile release from an emulsion: Headspace and in-mouth studies. *Journal of Agricultural and Food Chemistry*, *49* (2), 804-810.

Doyennette, M., Deleris, I., Saint-Eve, A., Gasiglia, A., Souchon, I., & Trelea, I. C. (2011). The dynamics of aroma compound transfer properties in cheeses during simulated eating conditions. *Food Research International*, *44* (10), 3174-3181.

Druaux, C., Lubbers, S., Charpentier, C., & Voilley, A. (1995). Effects of physicochemical parameters of a model wine on the binding of gamma-decalactone on bovine serum-albumin. *Food Chemistry*, *53* (2), 203-207.

Dufour, C., & Bayonove, C. (1999a). Interactions between wine polyphenols and aroma substances. An insight at the molecular level. *Journal of Agricultural and Food Chemistry*, *47* (2), 678-684.

Dufour, C., & Bayonove, C. L. (1999b). Influence of wine structurally different polysaccharides on the volatility of aroma substances in a model system. *Journal of Agricultural and Food Chemistry*, *47* (2), 671-677.

E

Ebeler (2005) En: Deibler *Handbook of Flavor Characterization*. CRC Press.

Ebeler, S. E., & Thorngate, J. H. (2009). Wine Chemistry and Flavor: Looking into the Crystal Glass. *Journal of Agricultural and Food Chemistry*, *57* (18), 8098-8108.

Engelen, L., de Wijk, R. A., Prinz, J. F., Janssen, A. M., Weenen, H., & Bosman, F. (2003). The effect of oral and product temperature on the perception of flavor and texture attributes of semi-solids. *Appetite*, *41* (3), 273-281.

Escalona, H., Homman-Ludiye, M., Piggott, J. R., & Paterson, A. (2001). Effect of potassium bitartrate, (+)-catechin and wood extracts on the volatility of ethyl hexanaote and octanal in ethanol/water solutions. *Lebensmittel-Wissenschaft und-Technologie*, *34* (2), 76-80.

Escudero, A., Gogorza, B., Melus, M. A., Ortin, N., Cacho, J., & Ferreira, V. (2004). Characterization of the aroma of a wine from Maccabeo. Key role played by compounds with low odor activity values. *Journal of Agricultural and Food Chemistry*, *52* (11), 3516-3524.

Escudero, A., Campo, E., Fariña, L., Cacho, J., & Ferreira, V. (2007). Analytical characterization of the aroma of five premium red wines. Insights into the role of odor families and the concept of fruitiness of wines. *Journal of Agricultural and Food Chemistry*, *55* (11), 4501-4510.

Espinosa-Diaz, M. (2004). Comparison between orthonasal and retronasal flavour perception at different concentrations. *Flavour and Fragrance Journal*, *19* (6), 499-504.

Ettre, L. S., Welter, C., & Kolb, B. (1993). Determination of gas-liquid partition coefficients by automatic equilibrium headspace-gas chromatography utilizing the phase ratio variation method. *Chromatographia*, *35* (1-2), 73-84.

Ezquerro, O., Pons, B., & Tena, M. T. (2003). Multiple headspace solid-phase microextraction for the quantitative determination of volatile organic compounds in multilayer packagings. *Journal of Chromatography A*, *999* (1-2), 155-164.

Ezquerro, O., Ortiz, G., Pons, B., & Tena, M. T. (2004). Determination of benzene, toluene, ethylbenzene and xylenes in soils by multiple headspace solid-phase microextraction. *Journal of Chromatography A*, *1035* (1), 17-22.

F

Feron, G., Ayed, C., Qannari, E. M., Courcoux, P., Laboure, H., & Guichard, E. (2014). Understanding Aroma Release from Model Cheeses by a Statistical Multiblock Approach on Oral Processing. *Plos One*, *9* (4).

Ferreira, V., Rapp, A., Cacho, J. F., Hastrich, H., & Yavas, I. (1993). Fast and quantitaive-determination of wine flavor compounds using microextraction with freon-113. Journal of Agricultural and Food Chemistry, 41 (9), 1413-1420.

Ferreira, V., López, R., Escudero, A., & Cacho, J. F. (1998). Quantitative determination of trace and ultratrace flavour active compounds in red wines through gas chromatographic-ion trap mass spectrometric analysis of microextracts. Journal of Chromatography A, 806 (2), 349-354.

Ferreira, V. (2002). ¿Puede el aroma del vino explicarse con una ecuación química? ACE *Revista de Enología* (24).

Ferreira, V., Ortin, N., Escudero, A., López, R., & Cacho, J. (2002a). Chemical characterization of the aroma of Grenache rose wines: Aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. *Journal of Agricultural and Food Chemistry*, 50 (14), 4048-4054.

Ferreira, V., Ortin, N., Escudero, A., Lopez, R., & Cacho, J. (2002). Chemical characterization of the aroma of Grenache rose wines: Aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. *Journal of Agricultural and Food Chemistry*, *50*(14), 4048-4054.

Ferreira, V., Juan, F. S., Escudero, A., Culleré, L., Fernández-Zurbano, P., Saenz-Navajas, M. P., & Cacho, J. (2009). Modeling quality of premium spanish red wines from gas chromatography-olfactometry data. *Journal of Agricultural and Food Chemistry*, *57* (16), 7490-7498.

Ferreira, V., & Cacho, J. (2009). Identification of Impact Odorants of Wines. En: M. V. Moreno-Arribas, & P. Carmen, *Wine Chemistry and Biochemistry*. (pp. 393-416): Springer.

Fiches, G., Deleris, I., Saint-Eve, A., Pollet, B., Brunerie, P., & Souchon, I. (2013). Modifying PTR-MS operating conditions for quantitative headspace analysis of hydro-alcoholic beverages. 1. Variation of the mean collision energy to control ionization processes occurring during PTR-MS analyses of 10-40% (v/v) ethanol-water solutions. *International Journal of Mass Spectrometry*, *356*, 41-45.

Fiches, G., Deleris, I., Saint-Eve, A., Brunerie, P., & Souchon, I. (2014). Modifying PTR-MS operating conditions for quantitative headspace analysis of hydro-alcoholic beverages. 2. Brandy characterization and discrimination by PTR-MS. *International Journal of Mass Spectrometry*, *360*, 15-23.

Frank, D., Appelqvist, I., Piyasiri, U., Wooster, T. J., & Delahunty, C. (2011). Proton Transfer Reaction Mass Spectrometry and Time Intensity Perceptual Measurement of Flavor Release from Lipid Emulsions Using Trained Human Subjects. *Journal of Agricultural and Food Chemistry*, *59* (9), 4891-4903.

Frasnelli, J., Van Ruth, S., Kriukova, I., & Hummel, T. (2005). Intranasal concentrations of orally administered flavors. *Chemical Senses*, *30* (7), 575.

Friel, E. N., & Taylor, A. J. (2001). Effect of salivary components on volatile partitioning from solutions. *Journal of Agricultural and Food Chemistry*, *49* (8), 3898-3905.

G

Genovese, A., Piombino, P., Gambuti, A., & Moio, L. (2009). Simulation of retronasal aroma of white and red wine in a model mouth system. Investigating the influence of saliva on volatile compound concentrations. *Food Chemistry*, *114* (1), 100-107.

Gierczynski, I., Guichard, E., & Laboure, H. (2011). Aroma perception in dairy products: the roles of texture, aroma release and consumer physiology. A review. *Flavour and Fragrance Journal*, *26* (3), 141-152.

Goldner, M. C., & Zamora, M. C. (2010). Effect of polyphenol concentrations on astringency perception and its correlation with gelatin index of red wine. *Journal of Sensory Studies*, *25*(5), 761-777.

Goodstein, B., Evans, Ross. (2014). Perception of flavor finish in model white wine: A time-intensity study. *Food Quality and Preference*, *36*, 50-60.

Grosch, W. (1993). Detection of potent odorants in foods by aroma extract dilution analysis. *Trends in Food Science & Technology*, *4* (3), 68-73.

Guinard, J. X., & Mazzucchelli, R. (1996). The sensory perception of texture and mouthfeel. *Trends in Food Science & Technology*, *7* (7), 213-219.

Guth, H. (1997). Identification of character impact odorants of different white wine varieties. *Journal of Agricultural and Food Chemistry*, *45* (8), 3022-3026.

H

Haahr, A. M., Bardow, A., Thomsen, C. E., Jensen, S. B., Nauntofte, B., Bakke, M., Adler-Nissen, J., & Bredie, W. L. P. (2004). Release of peppermint flavour compounds from chewing gum: effect of oral functions. *Physiology & Behavior*, *82* (2-3), 531-540.

Hansel, A., Jordan, A., Warneke, C., Holzinger, R., & Lindinger, W. (1998). Improved detection limit of the proton-transfer reaction mass spectrometer: On-line monitoring of volatile organic compounds at mixing ratios of a few pptv. *Rapid Communications in Mass Spectrometry*, *12* (13), 871-875.

Hansson, A., Giannouli, P., & Van Ruth, S. (2003). The influence of gel strength on aroma release from pectin gels in a model mouth and in vivo, monitored with proton-transfer-reaction mass spectrometry. *Journal of Agricultural and Food Chemistry*, *51* (16), 4732-4740.

Harrison, M. (1998). Effect of breathing and saliva flow on flavor release from liquid foods. *Journal of Agricultural and Food Chemistry*, *46* (7), 2727-2735.

Hartmann, P., McNair, H., & Zoecklein, B. (2002). Measurement of 3-alkyl-2-methoxypyrazine by headspace solid-phase microextraction in spiked model wines. *American Journal of Enology and Viticulture*, *53* (4), 285.

Heenan, S., Soukoulis, C., Silcock, P., Fabris, A., Aprea, E., Cappellin, L., Maerk, T. D., Gasperi, F., & Biasioli, F. (2012). PTR-TOF-MS monitoring of in vitro and in vivo flavour release in cereal bars with varying sugar composition. *Food Chemistry, 131* (2), 477-484.

Helmerhorst, E. J., & Oppenheim, F. G. (2007). Saliva: a dynamic proteome. *Journal of Dental Research, 86* (8), 680-693.

Hernandez-Orte, P., Cersosimo, M., Loscos, N., Cacho, J., Garcia-Moruno, E., & Ferreira, V. (2009). Aroma development from non-floral grape precursors by wine lactic acid bacteria. *Food Research International, 42* (7), 773-781.

Hernanz, D., Gallo, V., Recamales, A. F., Melendez-Martinez, A. J., & Heredia, F. J. (2008). Comparison of the effectiveness of solid-phase and ultrasound-mediated liquid-liquid extractions to determine the volatile compounds of wine. *Talanta, 76* (4), 929-935.

Hills, B. P., & Harrison, M. (1995). Two-film theory of flavour release from solids. *International Journal of Food Science and Technology, 30* (4), 425-436.

Hirtz, C., Chevalier, F., Centeno, D., Egea, J. C., Rossignol, M., Sommerer, N., & de Periere, D. (2005). Complexity of the human whole saliva proteome. *Journal of Physiology and Biochemistry, 61*(3), 469-480.

Hodgson, M., Linforth, R. S. T., & Taylor, A. J. (2003). Simultaneous real-time measurements of mastication, swallowing, nasal airflow, and aroma release. *Journal of Agricultural and Food Chemistry, 51* (17), 5052-5057.

Hosoi, A. E., & Bush, J. W. M. (2001). Evaporative instabilities in climbing films. *Journal of Fluid Mechanics, 442*, 217-239.

Hussein, M. M., Kachikian, R., & Pidel, A. R. (1983). Analysis for flavor residuals in the mouth by gas-chromatography. *Journal of Food Science, 48* (6), 1884-1885.

Humphrey, S. P., & Williamson, R. T. (2001). A review of saliva: Normal composition, flow, and function. *Journal of Prosthetic Dentistry, 85*(2), 162-169.

I

Ingham, K. E., Linforth, R. S. T., & Taylor, A. J. (1995). The effect of eating on aroma release from strawberries. *Food Chemistry, 54* (3), 283-288.

Inoue, T. (2008). *Diacetyl in fermented foods and beverages*.

J

Jakubovics, N. S., & Kolenbrander, P. E. (2010). The road to ruin: the formation of disease-associated oral biofilms. *Oral Diseases*, *16*(8), 729-739.

Jehmlich, N., Dinh, K. H. D., Gesell-Salazar, M., Hammer, E., Steil, L., Dhople, V. M., Schurmann, C., Holtfreter, B., Kocher, T., & Voelker, U. (2013). Quantitative analysis of the intra- and inter-subject variability of the whole salivary proteome. *Journal of Periodontal Research*, *48* (3), 392-403.

Jones, P. R., Gawel, R., Francis, I. L., & Waters, E. J. (2008). The influence of interactions between major white wine components on the aroma, flavour and texture of model white wine. *Food Quality and Preference*, *19* (6), 596-607.

Jung, D., de Ropp, J., & Ebeler, S. (2000). Study of interactions between food phenolics and aromatic flavors using one-and two-dimensional ^{1}H NMR spectroscopy. *Journal of Agricultural and Food Chemistry*, *48* (2), 407-412.

Jung, D. M., & Ebeler, S. E. (2003). Headspace solid-phase microextraction method for the study of the volatility of selected flavor compounds. *Journal of Agricultural and Food Chemistry*, *51* (1), 200-205.

Juteau, A., Cayot, N., Chabanet, C., Doublier, J. L., & Guichard, E. (2004). Flavour release from polysaccharide gels: different approaches for the determination of kinetic parameters. *Trends in Food Science & Technology*, *15*(7-8), 394-402.

K

Kamonpatana, K., Giusti, M. M., Chitchumroonchokchai, C., MorenoCruz, M., Riedl, K. M., Kumar, P., & Failla, M. L. (2012). Susceptibility of anthocyanins to ex vivo degradation in human saliva. *Food Chemistry*, *135* (2), 738-747.

King, B. M., Arents, P., Bouter, N., Duineveld, C. A. A., Meyners, M., Schroff, S. I., & Soekhai, S. T. (2006). Sweetener/sweetness-induced changes in flavor perception and flavor release of fruity and green character in beverages. *Journal of Agricultural and Food Chemistry*, *54* (7), 2671-2677.

King, E. S., Dunn, R. L., & Heymann, H. (2013). The influence of alcohol on the sensory perception of red wines. *Food Quality and Preference*, *28* (1), 235-243.

Kuo, Y. L., Pangborn, R. M., & Noble, A. C. (1993). Temporal patterns of nasal, oral, and retronasal perception of citral and vanillin and interaction of these odorants with selected tastants. *International Journal of Food Science and Technology*, *28*(2), 127-137.

L

Land, D. G. (1996). Perspectives on the effects of interactions on flavor perception: An overview. En: R. J. McGorrin, & J. V. Leland, *Flavor-Food Interactions*, vol. 633 (p. 2-11).

Landy, P., Druaux, C., & Voilley, A. (1995). Retention of aroma compounds by proteins in aqueous-solution. *Food Chemistry*, *54* (4), 387-392.

Langourieux, S., & Crouzet, J. (1997). Study of interactions between aroma compounds and glycopeptides by a model system. *Journal of Agricultural and Food Chemistry, 45* (5), 1873-1877.

Lasekan, O., Buettner, A., & Christlbauer, M. (2009). Investigation of the retronasal perception of palm wine (Elaeis guineensis) aroma by application of sensory analysis and exhaled odorant measurement (EOM). *African Journal of Food, Agriculture, Nutrition and Development*, *9* (2), 793-813.

Lasekan, O. (2013). A Comparative Analysis of the Influence of Human Salivary Enzymes on Odorant Concentration in Three Palm Wines. *Molecules*, *18* (10), 11809-11823.

Le Berre, E., Atanasova, B., Langlois, D., Etiévant, P., & Thomas-Danguin, T. (2007). Impact of ethanol on the perception of wine odorant mixtures. *Food Quality and Preference*, *18* (6), 901-908.

Lee, W. E., & Pangborn, R. M. (1986). Time-intensity - the temporal aspects of sensory perception. *Food Technology*, *40* (11), 71.

Li, Z. X., Lu, J. R., Styrkas, D. A., Thomas, R. K., Rennie, A. R., & Penfold, J. (1993). The structure of the surface of ethanol-water mixtures. *Molecular Physics*, *80*(4), 925-939.

Li, J., Helmerhorst, E. J., Leone, C. W., Troxler, R. F., Yaskell, T., Haffajee, A. D., Socransky, S. S., & Oppenheim, F. G. (2004). Identification of early microbial colonizers in human dental biofilm. *Journal of Applied Microbiology*, *97*(6), 1311-1318.

Lindinger, W., Hansel, A., & Jordan, A. (1998). On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS) medical applications, food control and environmental research. *International Journal of Mass Spectrometry and Ion Processes*, *173* (3), 191-241.

Linforth, R., & Taylor, A. (1993). Measurement of volatile release in the mouth. *Food Chemistry*, *48*, 115-120.

Linforth, R. S. T., Baek, I., & Taylor, A. J. (1999). Simultaneous instrumental and sensory analysis of volatile release from gelatine and pectin/gelatine gels. *Food Chemistry*, *65* (1), 77-83.

Linforth, R., & Taylor, A. J. (2000). Persistence of volatile compounds in the breath after their consumption in aqueous solutions. *Journal of Agricultural and Food Chemistry*, *48*(11), 5419-5423.

Linforth, R., Martin, F., Carey, M., Davidson, J., & Taylor, A. J. (2002). Retronasal transport of aroma compounds. *Journal of Agricultural and Food Chemistry*, *50* (5), 1111-1117.

Linforth, R., & Taylor, A. (2006). The process of flavour release. En: A. Voilley and P. Etievant, *Flavour in Food* (p. 287-307). New York: CRC Press.

Linforth, R., Cabannes, M., Hewson, L., Yang, N., & Taylor, A. (2010). Effect of Fat Content on Flavor Delivery during Consumption: an in Vivo Model. Journal of Agricultural and Food Chemistry, 58 (11), 6905-6911.

López, R., Aznar, M., Cacho, J., & Ferreira, V. (2002). Determination of minor and trace volatile compounds in wine by solid-phase extraction and gas chromatography with mass spectrometric detection. *Journal of Chromatography A, 966* (1-2), 167-177.

Lorrain, B., Tempere, S., Iturmendi, N., Moine, V., de Revel, G., & Teissedre, P.-L. (2013). Influence of phenolic compounds on the sensorial perception and volatility of red wine esters in model solution: An insight at the molecular level. *Food Chemistry*, *140* (1-2), 76-82.

Lubbers, S., Charpentier, C., Feuillat, M., & Voilley, A. (1994). Influence of yeast walls on the behavior of aroma compounds in a model wine. *American Journal of Enology and Viticulture*, *45* (1), 29.

Lubbers, S., Verret, C., & Voilley, A. (2001). The effect of glycerol on the perceived aroma of a model wine and a white wine. *LWT - Food Science and Technology*, *34* (4), 262-265.

Lubbers, S., & Butler, E. (2010). Effects of texture and temperature on the kinetic of aroma release from model dairy custards. *Food Chemistry, 123* (2), 345-350.

Lytra, G., Tempere, S., Le Floch, A., de Revel, G., & Barbe, J.-C. (2013). Study of Sensory Interactions among Red Wine Fruity Esters in a Model Solution. *Journal of Agricultural and Food Chemistry*, *61* (36), 8504-8513.

M

Maarse, H., & Visscher, C. A. (1989). *Volatile compounds in food and beverages*. The Netherlands, Zeist Grafische Industry Kreon.

Macakova, L., Yakubov, G. E., Plunkett, M. A., & Stokes, J. R. (2011). Influence of ionic strength on the tribological properties of pre-adsorbed salivary films. *Tribology International, 44*(9), 956-962.

Malone, M. E., Appelqvist, I. A. M., & Norton, I. T. (2003). Oral behaviour of food hydrocolloids and emulsions. Part 2. Taste and aroma release. *Food Hydrocolloids*, *17*(6), 775-784.

Margomenou, L., Birkmyre, L., Piggott, J. R., & Paterson, A. (2000). Optimisation and validation of the "strathclyde simulated mouth" for beverage flavour research. *Journal of the Institute of Brewing*, *106* (2), 101-105.

Marsh, P.D. (2009) Diseases caused by oral bacteria. In Food constituents and oral health:Currents status and future prospects ed Wilson, M. pp. 1-18 United Kingdom: HPA CEPR & Leeds Dental Institute

Martinez-Rodriguez, A. J., & Polo, M. C. (2000). Characterization of the nitrogen compounds released during yeast autolysis in a model wine system. *Journal of Agricultural and Food Chemistry*, *48*(4), 1081-1085.

Mayr, C. M., Parker, M., Baldock, G. A., Black, C. A., Pardon, K. H., Williamson, P. O., Herderich, M. J., & Francis, I. L. (2014). Determination of the Importance of In-Mouth Release of Volatile Phenol Glycoconjugates to the Flavor of Smoke-Tainted Wines. *Journal of Agricultural and Food Chemistry*, *62*(11), 2327-2336.

McDaniel, M., Miranda-López, R., Walson, B., Micheals, N., & Libbey, L. (1990). Pinot Noir aroma: a sensory/gas chromatographic approach. En: G. Charalambous, *Flavors and Off-Flavors* (p. 23-36). Amsterdam: Elsevier.

Mehinagic, E., Prost, C., & Demaimay, M. (2004). Optimization of extraction of apple aroma by dynamic headspace and influence of saliva on extraction of volatiles. *Journal of Agricultural and Food Chemistry*, *52* (16), 5175-5182.

Meillon, S., Urbano, C., & Schlich, P. (2009). Contribution of the Temporal Dominance of Sensations (TDS) method to the sensory description of subtle differences in partially dealcoholized red wines. *Food Quality and Preference*, *20* (7), 490-499.

Meillon, S., Dugas, V., Urbano, C., & Schlich, P. (2010). Preference and Acceptability of Partially Dealcoholized White and Red Wines by Consumers and Professionals. *American Journal of Enology and Viticulture*, *61* (1), 42-52.

Mese, H., & Matsuo, R. (2007). Salivary secretion, taste and hyposalivation. *Journal of Oral Rehabilitation*, *34* (10), 711-723.

Mestres, M., Busto, O., & Guasch, J. (1998). Headspace solid-phase microextraction analysis of volatile sulphides and disulphides in wine aroma. *Journal of Chromatography A*, *808* (1-2), 211-218.

Mestres, M., Kieffer, R., & Buettner, A. (2006). Release and perception of ethyl butanoate during and after consumption of whey protein gels: Relation between textural and physiological parameters. *Journal of Agricultural and Food Chemistry*, *54* (5), 1814-1821.

Mialon, V. S., & Ebeler, S. E. (1997). Time-intensity measurement of matrix effects on retronasal aroma perception. *Journal of Sensory Studies, 12* (4), 303-316.

Mishellany-Dutour, A., Renaud, J., Peyron, M.-A., Rimek, F., & Woda, A. (2008). Is the goal of mastication reached in young dentates, aged dentates and aged denture wearers? *British Journal of Nutrition, 99*(1), 121-128.

Mishellany-Dutour, A., Woda, A., Laboure, H., Bourdiol, P., Lachaze, P., Guichard, E., & Feron, G. (2012). Retro-Nasal Aroma Release Is Correlated with Variations in the In-Mouth Air Cavity Volume after Empty Deglutition. *Plos One, 7* (7).

Mitropoulou, A., Hatzidimitriou, E., & Paraskevopoulou, A. (2011). Aroma release of a model wine solution as influenced by the presence of non-volatile components. Effect of commercial tannin extracts, polysaccharides and artificial saliva. *Food Research International, 44* (5), 1561-1570.

Moio, L., Ugliano, M., Gambuti, A., Genovese, A., & Piombino, P. (2004). Influence of clarification treatment on concentrations of selected free varietal aroma compounds and glycoconjugates in Falanghina (Vitis vinifera L.) must and wine. *American Journal of Enology and Viticulture, 55* (1), 7-12.

Morell, P., Hernando, I., & Fiszman, S. M. (2014). Understanding the relevance of in-mouth food processing. A review of in vitro techniques. *Trends in Food Science & Technology, 35* (1), 18-31.

Muñoz-Gonzalez, C., Rodriguez-Bencomo, J. J., Moreno-Arribas, M. V., & Pozo-Bayon, M. A. (2011). Beyond the characterization of wine aroma compounds: looking for analytical approaches in trying to understand aroma perception during wine consumption. *Analytical and Bioanalytical Chemistry, 401* (5), 1497-1512.

N

Neyraud, E., Palicki, O., Schwartz, C., Nicklaus, S., & Feron, G. (2012). Variability of human saliva composition: Possible relationships with fat perception and liking. *Archives of Oral Biology, 57* (5), 556-566.

Neyraud, E. (2014). Role of saliva in oral food perception. *Monographs in oral science, 24*, 61-70.

Nicolazzo, J. A., Reed, B. L., & Finnin, B. C. (2005). Buccal penetration enhancers - How do they really work? *Journal of Controlled Release, 105*(1-2), 1-15.

Noble, A., & Bursick, G. (1984). The contribution of glycerol to perceived viscosity and sweetness in white wine. *American Journal of Enology and Viticulture, 35* (2), 110.

Noble, A. C. (1995). Application of time-intensity procedures for the evaluation of taste and mouthfeel. *American Journal of Enology and Viticulture, 46* (1), 128-133.

Normand, V., Avison, S., & Parker, A. (2004). Modeling the kinetics of flavour release during drinking. *Chem Senses*, *29*(3), 235-245.

Nunez, Y. P., Carrascosa, A. V., Gonzalez, R., Polo, M. C., & Martinez-Rodriguez, A. J. (2005). Effect of accelerated autolysis of yeast on the composition and foaming properties of sparkling wines elaborated by a champenoise method. *Journal of Agricultural and Food Chemistry*, *53* (18), 7232-7237.

Nurgel, C., & Pickering, G. (2005). Contribution of glycerol, ethanol and sugar to the perception of viscosity and density elicited by model white wines. *Journal of Texture Studies*, *36* (3), 303-323.

O

O'Riordan, P. J., & Delahunty, C. M. (2001). Comparison of volatile compounds released during the consumption of Cheddar cheese with compounds extracted by vacuum distillation using gas chromatography-olfactometry. *Flavour and Fragrance Journal*, *16* (6), 425-434.

Odake, S., Roozen, J. P., & Burger, J. J. (1998). Effect of saliva dilution on the release of diacetyl and 2-heptanone from cream style dressings. *Nahrung-Food*, *42* (6), 385-391.

Ortega-Heras, M., González-SanJosé, M. L., & Beltrán, S. (2002). Aroma composition of wine studied by different extraction methods. *Analytica Chimica Acta*, *458* (1), 85-93.

Overbosch, P. (1986). A theoretical-model for perceived intensity in human taste and smell as a function of time. *Chemical Senses*, *11* (3), 315-329.

Overbosch, P., Afterof, W. G. M., & Haring, P. G. M. (1991). Flavor release in the mouth. *Food Reviews International*, *7* (2), 137-184.

P

Pedersen, A. M., Bardow, A., Jensen, S. B., & Nauntofte, B. (2002). Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion. *Oral Diseases*, *8* (3), 117-129.

Perestrelo, R., Nogueira, J. M. F., & Cámara, J. S. (2009). Potentialities of two solventless extraction approaches-stir bar sorptive extraction and headspace solid-phase microextraction for determination of higher alcohol acetates, isoamyl esters and ethyl esters in wines. *Talanta*, *80* (2), 622-630.

Petrozziello, M., Asproudi, A., Guaita, M., Borsa, D., Motta, S., Panero, L., & Bosso, A. (2014). Influence of the matrix composition on the volatility and sensory perception of 4-ethylphenol and 4-ethylguaiacol in model wine solutions. *Food Chemistry*, *149*, 197-202.

Piggott, J., & Schaschkeb, C. (2001). Release cells, breath analysis and in-mouth analysis in flavour research. *Biomolecular Engineering*, *17* (4-5), 129-136

Pineau, B., Barbe, J. C., Van Leeuwen, C., & Dubourdieu, D. (2007). Which impact for b-damascenone on red wines aroma? *Journal of Agricultural and Food Chemistry*, *55* (10), 4103-4108.

Pineau, N., Schlich, P., Cordelle, S., Mathonniere, C., Issanchou, S., Imbert, A., Rogeaux, M., Etievant, P., & Koesterf, E. (2009). Temporal Dominance of Sensations: Construction of the TDS curves and comparison with time-intensity. *Food Quality and Preference*, *20* (6), 450-455.

Pionnier, E., Chabanet, C., Mioche, L., Le Quere, J. L., & Salles, C. (2004a). 1. In vivo aroma release during eating of a model cheese: Relationships with oral parameters. *Journal of Agricultural and Food Chemistry*, *52* (3), 557-564.

Pionnier, E., Chabanet, C., Mioche, L., Taylor, A. J., Le Quere, J. L., & Salles, C. (2004b). 2. In vivo nonvolatile release during eating of a model cheese: Relationships with oral parameters. *Journal of Agricultural and Food Chemistry*, *52* (3), 565-571.

Po, J. M. C., Kieser, J. A., Gallo, L. M., Tesenyi, A. J., Herbison, P., & Farella, M. (2011). Time-Frequency Analysis of Chewing Activity in the Natural Environment. *Journal of Dental Research*, *90*(10), 1206-1210.

Poette, J., Mekoue, J., Neyraud, E., Berdeaux, O., Renault, A., Guichard, E., Genot, C., & Feron, G. (2013). Fat sensitivity in humans: oleic acid detection threshold is linked to saliva composition and oral volume. *Flavour and Fragrance Journal*, *29* (1), 39-49.

Poinot, P., Arvisenet, G., Grua-Priol, J., Fillonneau, C., & Prost, C. (2009). Use of an artificial mouth to study bread aroma. *Food Research International*, *42* (5-6), 717-726.

Poinot, P., Arvisenet, G., Ledauphin, J., Gaillard, J.-L., & Prost, C. (2013). How can aroma-related cross-modal interactions be analysed? A review of current methodologies. *Food Quality and Preference*, *28*(1), 304-316.

Polásková, P., Herszage, J., & Ebeler, S. E. (2008). Wine flavor: chemistry in a glass. *Chemical Society Reviews*, *37* (12), 2798.

Pollien, P., Ott, A., Montigon, F., Baumgartner, M., Muñoz-Box, R., & Chaintreau, A. (1997). Hyphenated headspace-gas chromatography-sniffing technique: screening of impact odorants and quantitative aromagram comparisons. *Journal of Agricultural and Food Chemistry*, *45* (7), 2630-2637.

Pozo-Bayón, M. A., Pueyo, E., Martín-Álvarez, P. J., & Polo, M. C. (2001). Polydimethylsiloxane solid-phase microextraction-gas chromatography method for the analysis of volatile compounds in wines: Its application to the characterization of varietal wines. Journal of Chromatography A, 922 (1-2), 267-275.

Pozo-Bayón, M., & Reineccius, G. (2009). Interactions between wine matrix macro-components and aroma compounds. En: M. V. Moreno-Arribas, & P. Carmen, *Wine Chemistry and Biochemistry.* (pp. 417-435): Springer.

Pozo-Bayón, M., Santos, M., Martín-Álvarez, P., & Reineccius, G. (2009a). Influence of carbonation on aroma release from liquid systems using an artificial throat and a proton transfer reaction–mass spectrometric technique (PTR–MS). *Flavour and Fragrance Journal*, 24 (5), 226–233.

Pozo-Bayón, M. A., Andujar-Ortiz, I., Alcaide-Hidalgo, J. M., Martín-Álvarez, P. J., & Moreno-Arribas, M. V. (2009b). Characterization of commercial inactive dry yeast preparations for enological use based on their ability to release soluble compounds and their behavior toward aroma compounds in model wines. *Journal of Agricultural and Food Chemistry*, 57(22), 10784-10792.

Pozo-Bayón, M. A., Andújar-Ortiz, I., & Victoria Moreno-Arribas, M. (2009c). Volatile profile and potential of inactive dry yeast-based winemaking additives to modify the volatile composition of wines. J*ournal of the Science of Food and Agriculture*, 89 (10), 1665-1673.

Pozo-Bayón, M., Pimenta, P., Pilch, S., Masters, J., Martín-Álvarez, P., & Reineccius, G. (2010). API-IT-MS for measuring aroma release from dentifrice products using a device to simulate tooth brushing. *J. Agric. Food Chem*, *58* (8), 5034–5041.

Prinz, J. F., & Lucas, P. W. (1997). An optimization model for mastication and swallowing in mammals. *Proceedings of the Royal Society B-Biological Sciences*, *264* (1389), 1715-1721.

Pueyo, E., Dizy, M., & Polo, M. C. (1993). Varietal differentiation of must and wines by means of protein-fraction. *American Journal of Enology and Viticulture*, *44* (3), 255-260.

R

Rabe, S., Krings, U., Banavara, D. S., & Berger, R. G. (2002). Computerized apparatus for measuring dynamic flavor release from liquid food matrices. *Journal of Agricultural and Food Chemistry*, *50* (22), 6440-6447.

Rabe, S., Krings, U., & Berger, R. (2004b). In vitro study of the influence of physiological parameters on dynamic in-mouth flavour release from liquids. *Chemical Senses*, *29* (2), 153-162.

Rabe, S., Linforth, R., Krings, U., Taylor, A., & Berger, R. (2004c). Release from liquids: a comparison of in vivo APCI-MS, in-mouth headspace trapping and in vitro mouth model data. *Chemical Senses*, *29* (2), 163-173.

Ramirez, G. R., Lubbers, S., Charpentier, C., Feuillat, M., Voilley, A., & Chassagne, D. (2001). Aroma compound sorption by oak wood in a model wine. *J. Agric. Food Chem*, *49*(8), 3893-3897.

Ramirez, G., Chassagne, D., Feuillat, M., Voilley, A., & Charpentier, C. (2004). Effect of wine constituents on aroma compound sorption by oak wood in a model system. *American Journal of Enology and Viticulture*, *55* (1), 22-26.

Rapp, A., & Mandery, H. (1986). Wine aroma. *Experientia*, *42* (8), 873-884.

Reineccius G. (2006). En: Flavour Chemistry and Technology. New York, CRC Press.

Riberau-Gayon, Glories, Maujean, Dubosrdieu (2000) HandBook of Enology. Volume 2.Wiley.

Rickard, A. H., Gilbert, P., High, N. J., Kolenbrander, P. E., & Handley, P. S. (2003). Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends in Microbiology*, *11* (2), 94-100.

Roberts, D. D., & Acree, T. E. (1995). Simulation of retronasal aroma using a modified headspace technique - investigating the effects of saliva, temperature, shearing, and oil on flavor release. *Journal of Agricultural and Food Chemistry*, *43* (8), 2179-2186.

Roberts, D., & Acree, T. (1996). Effects of heating and cream addition on fresh raspberry aroma using a retronasal aroma simulator and gas chromatography olfactometry. *J. Agric. Food Chem.*, *44*(12), 3919–3925.

Robinson, A. L., Ebeler, S. E., Heymann, H., Boss, P. K., Solomon, P. S., & Trengove, R. D. (2009). Interactions between wine volatile compounds and grape and wine matrix components influence aroma compound headspace partitioning. *Journal of Agricultural and Food Chemistry*, *57* (21), 10313-10322.

Roozen, J., & Legger-Huysman, A. (1994). Sensory analysis and oral vapour gas chromatography of chocolate flakes. En: M. Rothe, & H. Kruse, *Aroma, perception, formation, evaluation* (p. 627- 632). Potsdam-Rehbrüke: Eigenverlag Deutsches Institut für Ernahrungsforschung.

Ross, C. F. (2009). Sensory science at the human-machine interface. *Trends in Food Science & Technology*, *20* (2), 63-72.

Roudnitzky, N., Bult, J. H. F., de Wijk, R. A., Reden, J., Schuster, B., & Hummel, T. (2011). Investigation of interactions between texture and ortho- and retronasal olfactory stimuli using psychophysical and electrophysiological approaches. *Behavioural Brain Research*, *216* (1), 109-115.

Ruijschop, R. M. A. J., Burgering, M. J. M., Jacobs, M. A., & Boelrijk, A. E. M. (2009). Retro-Nasal Aroma Release Depends on Both Subject and Product Differences: A Link to Food Intake Regulation? *Chemical Senses*, *34* (5), 395-403.

S

Sáenz-Navajas, M., Campo, E., Fernández-Zurbano, P., Valentin, D., & Ferreira, V. (2010). An assessment of the effects of wine volatiles on the perception of taste and astringency in wine. *Food Chemistry*, *121* (4), 1139-1149.

Salles, C., Tarrega, A., Mielle, P., Maratray, J., Gorria, P., Liaboeuf, J., & Liodenot, J. J. (2007). Development of a chewing simulator for food breakdown and the analysis of in vitro flavor compound release in a mouth environment. *Journal of Food Engineering*, *82* (2), 189-198.

Salles, C., Chagnon, M.-C., Feron, G., Guichard, E., Laboure, H., Morzel, M., Semon, E., Tarrega, A., & Yven, C. (2011). In-Mouth Mechanisms Leading to Flavor Release and Perception. *Critical Reviews in Food Science and Nutrition*, *51* (1), 67-90.

Schipper, R. G., Silletti, E., & Vinyerhoeds, M. H. (2007). Saliva as research material: Biochemical, physicochemlical and practical aspects. *Archives of Oral Biology*, *52*(12), 1114-1135.

Slomiany, B. L., Murty, V. L. N., Piotrowski, J., & Slomiany, A. (1996). Salivary mucins in oral mucosal defense. *General Pharmacology*, *27*(5), 761-771.

Soukoulis, C., Cappellin, L., Aprea, E., Costa, F., Viola, R., Maerk, T. D., Gasperi, F., & Biasioli, F. (2013). PTR-ToF-MS, A Novel, Rapid, High Sensitivity and Non-Invasive Tool to Monitor Volatile Compound Release During Fruit Post-Harvest Storage: The Case Study of Apple Ripening. *Food and Bioprocess Technology*, *6* (10), 2831-2843.

Spanel, P., & Smith, D. (1999). Selected ion flow tube - Mass spectrometry: Detection and real-time monitoring of flavours released by food products. *Rapid Communications in Mass Spectrometry*, *13*(7), 585-596.

Spielman, A. I. (1990). Interaction of saliva and taste. *Journal of Dental Research*, *69* (3), 838-843.

Spitaler, R., Araghipour, N., Mikoviny, T., Wisthaler, A., Via, J. D., & Märk, T. D. (2007). PTR-MS in enology: Advances in analytics and data analysis. *International Journal of Mass Spectrometry*, *266* (1-3), 1-7.

Starkenmann, C., Le Calve , B., Niclass, Y., Cayeux, I., Beccucci, S., & Troccaz, M. (2008). Olfactory perception of Cysteine- S-Conjugates from fruits and vegetables. *Journal of Agricultural and Food Chemistry*, *56*(20), 9575-9580.

Stephan, A., Bucking, M., & Steinhart, H. (2000). Novel analytical tools for food flavours. *Food Research International*, *33* (3-4), 199-209.

Styger, G., Prior, B., & Bauer, F. F. (2011). Wine flavor and aroma. *Journal of Industrial Microbiology & Biotechnology*, *38* (9), 1145-1159.

7

Taylor, A. J. (1996). Volatile flavor release from foods during eating. *Critical Reviews in Food Science and Nutrition*, *36* (8), 765-784.

Taylor, A. J., & Linforth, R. S. T. (2000). Techniques for measuring volatile release in vivo during consumption of food. En: D. D. Roberts, & A. J. Taylor, *Flavour release*, vol. 763 (p. 8-21). Washington D.C.: ACS Publications.

Taylor, A. (2002). Release and transport of flavors in vivo: physicochemical, physiological, and perceptual considerations. *Comprehensive Reviews in Food Science and Food Safety, 1* (2), 45-57.

Taylor, A. J., & Linforth, R. S. T. (2003). Direct mass spectrometry of complex volatile and non-volatile flavour mixtures. *International Journal of Mass Spectrometry*, *223-224*, 179-191.

Tian, Y., He, X., Torralba, M., Yooseph, S., Nelson, K. E., Lux, R., McLean, J. S., Yu, G., & Shi, W. (2010). Using DGGE profiling to develop a novel culture medium suitable for oral microbial communities. *Molecular Oral Microbiology*, *25*(5), 357-367.

Tromelin, A., Andriot, I., Kopjar, M., & Guichard, E. (2010). Thermodynamic and Structure Property Study of Liquid-Vapor Equilibrium for Aroma Compounds. *Journal of Agricultural and Food Chemistry*, *58* (7), 4372-4387.

Tsachaki, M., Linforth, R. S. T., & Taylor, A. J. (2005). Dynamic headspace analysis of the release of volatile organic compounds from ethanolic systems by direct APCI-MS. *Journal of Agricultural and Food Chemistry*, *53* (21), 8328-8333.

Tsachaki, M., Gady, A.-L., Kalopesas, M., Linforth, R. S. T., Athes, V., Marin, M., & Taylor, A. J. (2008). Effect of ethanol, temperature, and gas flow rate on volatile release from aqueous solutions under dynamic headspace dilution conditions. *Journal of Agricultural and Food Chemistry*, *56* (13), 5308-5315.

Tsachaki, M., Linforth, R. S. T., & Taylor, A. J. (2009). Aroma Release from Wines under Dynamic Conditions. *Journal of Agricultural and Food Chemistry*, *57* (15), 6976-6981.

Tsevdou, M., Soukoulis, C., Cappellin, L., Gasperi, F., Taoukis, P. S., & Biasioli, F. (2013). Monitoring the effect of high pressure and transglutaminase treatment of milk on the evolution of flavour compounds during lactic acid fermentation using PTR-ToF-MS. *Food Chemistry*, *138* (4), 2159-2167.

U

Ullrich, F., & Grosch, W. (1987). Identification of the most intense volatile flavour compounds formed during autoxidation of linoleic acid. *Zeitschrift fur Lebensmittel-Untersuchung und -Forschung*, *184* (4), 277-282.

V

Van Ruth, S., Roozen, J., & Cozijnsen, J. (1994). Comparison of dynamic headspace mouth model systems for flavour release from rehydrated bell pepper cuttings. En: H. Maarse, & D. Van Der Heij, *Trends in Flavor Research.* (p. 59-64). Amsterdam: Elsevier Science.

Van Ruth, S. M., Roozen, J. P., Nahon, D. F., Cozijnsen, J. L., & Posthumus, M. A. (1996). Flavour release from rehydrated French beans (Phaseolus vulgaris) influenced by composition and volume of artificial saliva. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung*, *203* (1), 1-6.

Van Ruth, S., & Roozen, J. (2000). Influence of mastication and saliva on aroma release in a model mouth system. *Food Chemistry*, *713*, 339-345.

Van Ruth, S. M., Grossmann, I., Geary, M., & Delahunty, C. M. (2001). Interactions between artificial saliva and 20 aroma compounds in water and oil model systems. *Journal of Agricultural and Food Chemistry*, *49* (5), 2409-2413.

Van Ruth, S. M. (2004). Evaluation of two gas chromatography-olfactometry methods: The detection frequency and perceived intensity method. *Journal of Chromatography A*, *1054* (1-2), 33-37.

Ventanas, S., Puolanne, E., & Tuorila, H. (2010). Temporal changes of flavour and texture in cooked bologna type sausages as affected by fat and salt content. *Meat Science*, *85* (3), 410-419.

Vidal, S., Williams, P., Doco, T., Moutounet, M., & Pellerin, P. (2003). The polysaccharides of red wine: total fractionation and characterization. *Carbohydrate Polymers*, *54* (4), 439-447.

Villamor, R. R., & Ross, C. F. (2013). Wine matrix compounds affect perception of wine aromas. *Annual Review of Food Science and Technology*, *4*, 1-20.

Villamor, R. R., Evans, M. A., & Ross, C. F. (2013). Effects of ethanol, tannin, and fructose concentrations on sensory properties of model red wines. *American Journal of Enology and Viticulture*, *64*(3), 342-348.

Voilley, A., Beghin, V., Charpentier, C., & Peyron, D. (1991). Interactions between aroma substances and macromolecules in a model wine. *Lebensmittel-Wissenschaft Technologie*, *24* (5), 469-472.

Voilley A. (2006). En: A. Voilley and P. Etievant, *Flavour in Food* (p. 117-129). New York: CRC Press.

Voirol, E., & Daget, N. (1986). Comparative-study of nasal and retronasal olfactory perception. *Lebensmittel-Wissenschaft & Technologie*, *19* (4), 316-319.

W

Walle, T., Browning, A. M., Steed, L. L., Reed, S. G., & Walle, U. K. (2005). Flavonoid glucosides are hydrolyzed and thus activated in the oral cavity in humans. *Journal of Nutrition*, *135* (1), 48-52.

Warneke, C., Rosen, S., Lovejoy, E., De Gouw, J., & Fall, R. (2004). Two additional advantages of proton transfer ion trap mass spectrometry. *Rapid Communications in Mass Spectrometry*, *18* (1), 133-134.

Warneke, C., De Gouw, J., Lovejoy, E., Murphy, P., Kuster, W., & Fall, R. (2005). Development of proton-transfer ion trap-mass spectrometry: on-line detection and identification of volatile organic compounds in air. *Journal of the American Society for Mass Spectrometry*, *16* (8), 1316-1324.

Weel, K. G. C., Boelrijk, A. E. M., Burger, J. J., Claassen, N. E., Gruppen, H., Voragen, A. G. J., & Smit, G. (2003). Effect of whey protein on the in vivo release of aldehydes. *Journal of Agricultural and Food Chemistry*, *51* (16), 4746-4752.

Weel, K. G. C., Boelrijk, A. E. M., Burger, J. J., Verschueren, M., Gruppen, H., Voragen, A. G. J., & Smit, G. (2004). New device to simulate swallowing and in vivo aroma release in the throat from liquid and semiliquid food systems. *Journal of Agricultural and Food Chemistry*, *52* (21), 6564-6571.

Whiton, R. S., & Zoecklein, B. W. (2000). Optimization of headspace solid-phase microextraction for analysis of wine aroma compounds. *American Journal of Enology and Viticulture*, *51* (4), 379-382.

Will, F., Pfeifer, W., & Dietrich, H. (1991). The importance of colloids for wine quality. *Wein-Wissenschaft*, *46* (4), 78-84.

Y

Yeretzian, C., Jordan, A., Brevard, H., & Lindinger, W. (2000). Time-resolved headspace analysis by proton-transfer-reaction mass-spectrometry. In: D. D. Roberts, & A. J. Taylor, *Flavour release*, vol. 763 (p. 58-72). Washington D.C.: ACS Publications.

Yven, C., Guessasma, S., Chaunier, L., Della Valle, G., & Salles, C. (2010). The role of mechanical properties of brittle airy foods on the masticatory performance. *Journal of Food Engineering*, *101* (1), 85-91.

Z

Zamora, M. C., Goldner, M. C., & Galmarini, M. V. (2006). Sourness-sweetness interactions in different media: white wine, ethanol and water. *Journal of Sensory Studies*, *21*, 601-611.

Anexos